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DISSERTAÇÃO

FRANCISCO MOURO

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2012

"Reza a tradição bíblica que a ausência de trabalho - a ociosidade - era a condição de felicidade do primeiro homem antes da sua queda. O amor à ociosidade permaneceu também no homem caído, mas a maldição continua a pesar sobre o homem, e não só porque temos de ganhar o nosso pão com o suor do rosto, mas também porque, pelas nossas características morais, não podemos ser ociosos e calmos. Uma voz secreta diz-nos que devemos sentir culpa da nossa ociosidade. Se o homem pudesse achar um estado em que, sendo ocioso, se sentisse útil e ciente do dever cumprido, acharia uma das facetas da felicidade primitiva."

Lev Tolstói, Guerra e Paz, 1869

"Não gostaria que alguém adoptasse o meu modo de vida por motivo nenhum; pode ocorrer que antes que o aprenda, eu já tenha descoberto outro para mim, e além disso desejo que haja no mundo tanto quanto possível pessoas diferentes. Gostaria, sim, que cada um se empenhasse em descobrir e seguir seu próprio caminho, em vez do trilhado pelo seu pai, sua mãe ou seu vizinho. Que o jovem construa, plante ou viaje, contanto que não seja impedido de fazer aquilo que, segundo ele, gostaria de fazer."

Henry David Thoreau, Walden, 1854

RESUMO

O hipocampo é a estrutura cerebral mais estudada na investigação neurocientífica. A subdivisão CA1 do hipocampo contém a população neuronal que apresenta menor heterogeneidade entre neurónios, encontrando-se organizada em camadas bem definidas, o que torna os neurónios fáceis de identificar e de estudar (Szilágyi *et al*. 2011).

O neurónio é a unidade sinalizadora individual do sistema nervoso central (SNC). Os neurónios estão inseridos numa complexa rede de circuitos neuronais que se encontra distribuída por todo o cérebro, comunicando entre si através de sinais eléctricos designados como potenciais de acção e potenciais sinápticos (Adrian, 1957).

A transmissão de informação entre um neurónio localizado pré-sinapticamente e uma célula pós-sináptica designa-se como transmissão sináptica (Breathnach, 2005). As sinapses químicas envolvem a libertação pré sináptica de neurotransmissores para a fenda sináptica (Connors & Long, 2004), activando receptores específicos localizados na membrana da célula pós-sináptica (Schoepp *et al.* 1999).

Os receptores NMDA (*N*-methyl-D-aspartate) são receptores ionotrópicos cujo principal agonista endógeno é o glutamato (Bakkar *et al.* 2011. As suas principais características são a dependência de voltagem, elevada permeabilidade ao cálcio e cinética de activação/desactivação lenta (Doherty & Sladek, 2011). Os receptores NMDA são moléculas heteroméricas formadas pelas subunidades GluN1, GluN2 e GluN3 (Groc *et al.* 2009). Na região CA1 do hipocampo, são tetra heteromeros compostos por duas subunidades GluN1 e duas subunidades GluN2 (Bakkar *et al*. 2011), podendo classificar-se como sinápticos ou extrasinápticos (Groc *et al*. 2009).

A actividade dos receptores NMDA está associada, de forma paradoxal, a fenómenos indutores de sobrevivência neuronal e de plasticidade sináptica (Larkman & Jack, 1995; Lipton & Nakanishi, 1999), e a episódios neurodegenerativos associados a morte neuronal (Xu *et al.* 2009). A ambivalência nas consequências da actividade dos receptores NMDA recebe o nome de "paradoxo dos receptores NMDA" (Hardingham & Bading, 2010), sendo atribuída à localização sináptica destes receptores. De facto, a actividade de receptores NMDA sinápticos aparenta estar associada a fenómenos de protecção neuronal, ao passo que a actividade de

receptores NMDA extrasinápticos conduz a fenómenos de apoptose neuronal (Hardingham & Bading, 2010; Stark & Bazan, 2011).

A actividade de receptores NMDA pode ser influenciada pela acção de neuromoduladores do SNC. A neuromodulação consiste na habilidade neuronal para modificar as propriedades eléctricas dos neurónios em resposta a mudanças bioquímicas intracelulares resultantes de estímulos sinápticos ou hormonais, permitindo ao SNC adaptar a sua capacidade de controlar funções fisiológicas num ambiente em constante mudança (Kaczmarek & Levitan, 1987).

A adenosina é um neuromodulador que medeia os seus efeitos biológicos através de quatros subtipos de receptores: receptores A_1, A_{2A}, A_{2B} e A_3 (A_1Rs, A_{2A}Rs, A_{2B}Rs eA_3Rs) (Tsutsui *et al* 2004). Grande parte das acções neuromoduladoras da adenosina são mediadas pelos receptores A_1 e A_{2A} (Gomes *et al.* 2010). Os receptores A_1 ligam-se a proteínas Gi, cuja actividade inibe a actividade da adenilato ciclase (van Calker *et al.* 1979). Por seu turno, os receptores A_{2A} ligam-se a proteínas Gs, que estimulam a expressão de adenilato ciclase (Corvol *et al*. 2001). Enquanto os receptores A_1 se encontram amplamente disseminado pelo cérebro (Dunwiddie & Masino, 2001), os receptores A_{2A} estão distribuídos em áreas especificas como o estriado (Haas & Selbach, 2000), embora estejam também presentes em outras áreas cerebrais (Fredholm *et al*. 2005).

A adenosina é uma substância neuromoduladora capaz de inibir ou de estimular a neurotransmissão no SNC (Fredholm & Dunwiddie, 1988). Os receptores A_1 reduzem a libertação de neurotransmissor e inibem a transmissão sináptica. As acções inibitórias mais proeminentes dos receptores A_1 estão identificadas em sistemas de transmissão glutamatérgica excitatória, através da inibição na libertação do neurotransmissor glutamato (Barrie & Nicholls, 1993).

Apesar da actividade dos receptores A_2 ter um impacto limitado no controlo da transmissão sináptica basal, eles são cruciais no controlo da plasticidade sináptica (Gomes *et al.* 2010). No hipocampo, a utilização de agonistas (Cunha *et al.* 1996) e antagonistas (Li & Henry, 1998) específicos para estes receptores provocam, respectivamente, aumentos e reduções na transmissão sináptica excitatória. A nível pré-sináptico, os receptores A_{2A} desempenham um papel facilitatório na libertação de glutamato (Lopes *et al.* 1999). No hipocampo, os efeitos moduladores dos receptores A_{2A} sobre a actividade dos receptores NMDA é menos bem conhecida. Noutras estruturas cerebrais há evidências de que, a nível pós-sináptico, os receptores A_{2A}

modulam a actividade dos receptores NMDA (Wirkner *et al.* 2004; Tebano *et al.* 2005; Rebola *et al.* 2008). De igual forma, sabe-se que em neurónios CA1 piramidais, os receptores A_{2A} de adenosina controlam de forma directa a activação de receptores AMPA de glutamato (Dias *et al.* 2002).

Este trabalho foi desenvolvido com o objectivo de compreender se a activação farmacológica de receptores A_{2A} de adenosina pode modular a actividade de receptores NMDA na região CA1 do hipocampo.

Utilizando fatias de hipocampo obtidas a partir de ratos Wistar jovem-adultos, os dados foram adquiridos através de técnicas de electrofisiologia, nomeadamente, procedimentos de *patch-clamp* utilizando a configuração *whole-cell*. Primeiramente, foi necessário garantir que as correntes evocadas refletiam exclusivamente actividade de receptores NMDA. Assim sendo, foi utilizado CNQX (10μM), um antagonista competitivo selectivo para receptores de glutamato AMPA e kainato. Os resultados não demonstram alterações significativas nas correntes (99%±2.9% n=3, $p>0.05$), o que permite concluir que as correntes registadas estavam apenas a ser evocadas pela actividade dos receptores NMDA. De igual forma, para garantir que este efeito está dependente da activação de receptores NMDA, foi utilizado um antagonista especifico destes receptores. A aplicação de DL-APV (50μM) provocou um decréscimo muito significativo nas correntes registadas (76%±4.9% n=3, $p<0.005$).

De seguida, com o objectivo de tentar encontrar um efeito modulador dos receptores A_{2A} sobre a actividade dos receptores NMDA, começou-se por avaliar os efeitos do CGS 21680, um agonista selectivo dos receptores A_{2A}, sobre a actividade dos receptores NMDA. A adição de CGS 21680 (30nM) provocou um aumento significativo nas correntes mediadas pela actividade dos receptores NMDA (23%±4,7% n=6, $p<0.005$). Estes dados apontam para um efeito modulador dos receptores A_{2A} que potencia a actividade dos receptores NMDA em células CA1 do hipocampo.

Para assegurar que este efeito reflecte um efeito modulador dos receptores A_{2A}, foi adicionado o agonista CGS 21680 na presença prévia do antagonista selectivo dos receptores A_{2A}, o SCH 58261. A adição de SCH 58261 (100nM) não causou diferenças significativas na amplitude das correntes (3%±14,5% n=3, $p>0.05$), o que significa que os receptores A_{2A} endógenos não contribuem para o efeito anteriormente registado. A adição subsequente do agonista CGS 21680 (30nM), após a adição prévia do antagonista durante 10 minutos, não provocou alterações

significativas na amplitude das correntes registadas (2%±9,0% n=5, p>0.05). O facto do efeito potenciador do agonista ser prevenido pela presença do antagonista dos receptores A_{2A}, permite concluir que este efeito modulador é mediado por receptores A_{2A}.

Estes resultados sugerem que os receptores A_{2A} modulam a actividade dos receptores NMDA, resultando numa potenciação das correntes pós-sinápticas mediadas pelos receptores NMDA. Estes resultados reflectem um efeito ainda não descrito das capacidades neuromoduladoras dos receptores A_{2A}.

Futuramente, seria importante discriminar se as correntes mediadas reflectem a actividade de receptores NMDA localizados sinapticamente ou extrasinapticamente, uma vez que a actividade de receptores NMDA localizados em diferentes áreas sinápticas tem implicações diferentes para a saúde neuronal. A memantina, um fármaco que bloqueia preferencialmente a actividade de receptores NMDA extrasinápticos, poder-se-á revelar uma ferramenta útil para uma investigação futura sobre o tema.

Palavras-chave: Adenosina, Hipocampo, Neuromodulação, Receptores NMDA,

ABSTRACT

Hippocampal excitatory synaptic plasticity is often considered the synaptic basis for memory formation. NMDAR activity is deeply involved in long-lasting changes in synaptic plasticity. Adenosine modulatory actions upon excitatory glutamatergic transmission are well described. However, the modulatory actions of adenosine A_{2A}Rs upon NMDARs activity in CA1 pyramidal cells have never been reported. Thus, the effect of A_{2A}R activation on NMDARs-mediated postsynaptic currents (PSCs) was examined in CA1 pyramidal neurons of young (3-10 weeks) rat hippocampal slices, by using the whole-cell patch-clamp technique (Vh = -60mV). NMDARs-mediated currents were evoked through pressure application of NMDA (150μM) (selective NMDAR agonist) directly onto the cell soma. Bath application of A_{2A}R agonist CGS 21680 (30nM) induced significant increases on NMDA-evoked currents (23%±4,7% n=6, p<0.005). To further address if this effect was caused by an A_{2A}R-mediated modulation of NMDA-evoked PSCs, CGS 21680 (30nM) was superfused in the presence of SCH 58261 (100nM), a selective A_{2A}R antagonist, which was added to the perfusion 10 minutes before the agonist. NMDA-evoked PSCs were not significantly altered by the presence of CGS 21680 (30nM) when A_{2A}Rs were previously blocked by SCH 58261 (100nM) (2%±9,0% n=5, p>0.05). To assure that the measured currents were elicited by NMDARs activity, CNQX (10μM) was used to block the activity of AMPA/kainate receptors. The results show no significant changes in NMDA-evoked PSCs in the presence of CNQX (99%±2.9% n=3, p>0.05), suggesting that these currents were mediated by NMDARs. Finally, the use of DL-APV (50μM) - selective NMDARs antagonist - served to further assure that NMDARs mediated the observed currents. In the presence of DL-APV, NMDA-evoked postsynaptic currents significantly decreased (76%±4.9% n=3, p<0.005). Together these results allow to conclude that A_{2A}Rs exert a modulatory effect over NMDARs activity at the CA1 neurons of the hippocampus, resulting in potentiation of NMDA-evoked PSCs.

Key Words: Adenosine, Hippocampus, NMDA receptor, Neuromodulation

LIST OF ABBREVIATIONS

A_1R - adenosine A_1 receptors

$A_{2A}R$ – adenosine A_{2A} receptors

A_3R – adenosine A_3 receptors

$A_{2B}R$ – adenosine A_{2B} receptors

aCSF – Artificial Cerebrospinal Fluid

ADA – Adenosine deaminase

Ag – Antigen

AMPA – α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

ATP – Adenosine triphosphate

BDNF –Brain-derived neurotropic factor

CA1-CA3 – Cornu Ammonis, areas 1-3

CAM IV – Calmodulin-dependent protein

cAMP – Cyclic Adenosine Monophosphate

CGS 21680 – 2-[4-(2-p-carboxyethyl) phenylamino]-5'N-ethylcarbozamidoadenosine

CNQX – -cyano-7-nitroquinoxaline-2,3-dione

CNS – Central Nervous System

CREB – cAMP response element binding

CPA – N6-cyclopentyladenosine

DG – Dentate Gyrus

DL-AP5 – DL-2-Amino-5-phosphonopentanoic acid

DMSO – Dimethyl sulfoxide

DPCPX – 1,3-dipropyl-8-cyclopentylxanthine

EC – Entorhinal Cortex

FOXO – Forkhead protein family of transcription factors

GABA – γ-Aminobutiric Acid

LTP – Long-term-potentiation

MAGUKS – Membrane-associated guanylate kinases

mGluR5 – Metabotropic glutamate receptor 5

NMDA – N-methyl-D-aspartic acid

NMDAR – N-methyl-D-aspartate receptor

PKA – Protein Kinase A

PSD-95 – postsynaptic density protein 95

PUMA - p53 upregulated modulator of apoptosis

ROS – Reactive oxygen species

Sb – Subiculum

SC – Schaffer Collateral

SCH 58261 - 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5 c]pyrimidine

SEM – Standard Error of the Mean

Trp53 – Transformation related protein 53

TTX – Tetrodotoxin

Vh – Voltage hold

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AIMS

1

The present work was designed with the main objective of identifying a possible modulatory action of adenosine A_{2A} receptor upon NMDA receptor activity in CA1 pyramidal cells. The inhibitory modulatory role of adenosine A_1 receptors has already been well described and studied. However, the adenosinergic excitatory actions are yet to be fully understood. NMDA receptors located at the CA1 hippocampal area are key components in several forms of synaptic plasticity and are involved in memory consolidation and spatial learning. Comprehending how adenosine A_{2A} receptors modulate the activity of NMDA receptors in CA1 pyramidal cells can contribute to understand how adenosine modulates synaptic plasticity and memory.

STATE OF THE ART

2

2.1 - Hippocampus – structure, populations and neuronal circuitry

The hippocampus is, nowadays, the most widely studied brain structure in the nervous system. The interest in its study started more than a century ago sparked by the discovery of a prominent relationship between declarative memory and structures in the human medial temporal lobe, specifically the hipocampal formation (van Strien, 2009). Scoville and Milner's were the first investigators to address memory loss in humans following removal of the hippocampal region, showing in first hand that hippocampal structures are dedicated to memory functions, independent of other cognitive abilities. Besides that, even within memory, the role of the hippocampus is selective, both concerning time window and particular domain of memory processing (Eichenbaum, 2000). The ability to repeat or recognize items just brought into consciousness, in other words the immediate memory, is intact in patients with damaged hippocampal regions (Squirel *et al.* 1993; Corkin, 1984). Also, general world knowledge and ancient childhood memories doesn't seem to be impaired. These early findings suggested that the hippocampal regions play critical roles between the initial formation of memories and their final repository in the brain

(Eichenbaum, 2000). Specifically, the hippocampus plays an unavoidable role in the expression of autobiographical episodic and spatial memories (Nadel & Moscovitch, 1997).

The human ability to learn, store and recover information about daily unique and personal events is possible due to episodic memory. Generally, these memories include information about the location and time of a specific event, as well as very detailed characterization of the event itself (Dickerson & Eichenbaum, 2010). The hippocampus is widely evolved in episodic memory in both humans and animals, playing a crucial role in the functions of this particular type of memory (Deshmukh & Knierim, 2011). A vast and complex network of brain areas supports episodic memory, including widespread neocortical association areas and components of the medial temporal lobe, in which parahippocampal cortical areas and the hippocampus are included. The general organization of this neurocircuitry system is that virtually all neocortical association areas send projections that converge onto the parahippocampal areas surrounding the hippocampus. In turn, these parahippocampal areas send projections to all the subdivisions of the hippocampus (Dickerson & Eichenbaum, 2010), through a serial and unidirectional circuit that internally connects the hippocampal subdivisions. This circuit starts in the dentate gyrus and passes, subsequently and continuously, across CA1, CA3 and subiculum neuronal populations.

The most important component in the hippocampal circuitry consists in the trisynaptic loop, comprising the dentate gyrus, CA3 and CA1 neuron populations. The inflow of impulses that arrives from the entorhinal cortex and parahippocampal regions is propagated by excitatory synaptic transmissions that pass through the dentate gyrus and subsequently CA3 and CA1 regions. This information is then sent back to the entorhinal cortex areas, hence the trisynaptic loop (Wojtowicz, 2011). In the hippocampus the circuitry system is extremely important. Therefore, knowledge about hippocampal function is based upon neuronal interaction within this circuitry (Wojtowicz, 2011). Both lateral and medial areas of the entorhinal cortex separately send projections and information to all four hippocampal sub regions (Dickerson & Eichenbaum, 2010).

The various functions of the components in the trisynaptic loop are studied in the standard hippocampal slice preparation, which allows the possibility to work with really thin slices of tissue where all three hippocampal regions and most of their

synaptic interconnections are visible, as it is possible to observe in figure 2.1.1 (Andersen *et al.* 1971). The CA1 subdivision of the hippocampus contains the most studied neuronal population. This population shows less heterogeneity and the pyramidal neuronal cells are organized in well-defined layers that are easy to identify and study (Szilágyi *et al*. 2011).

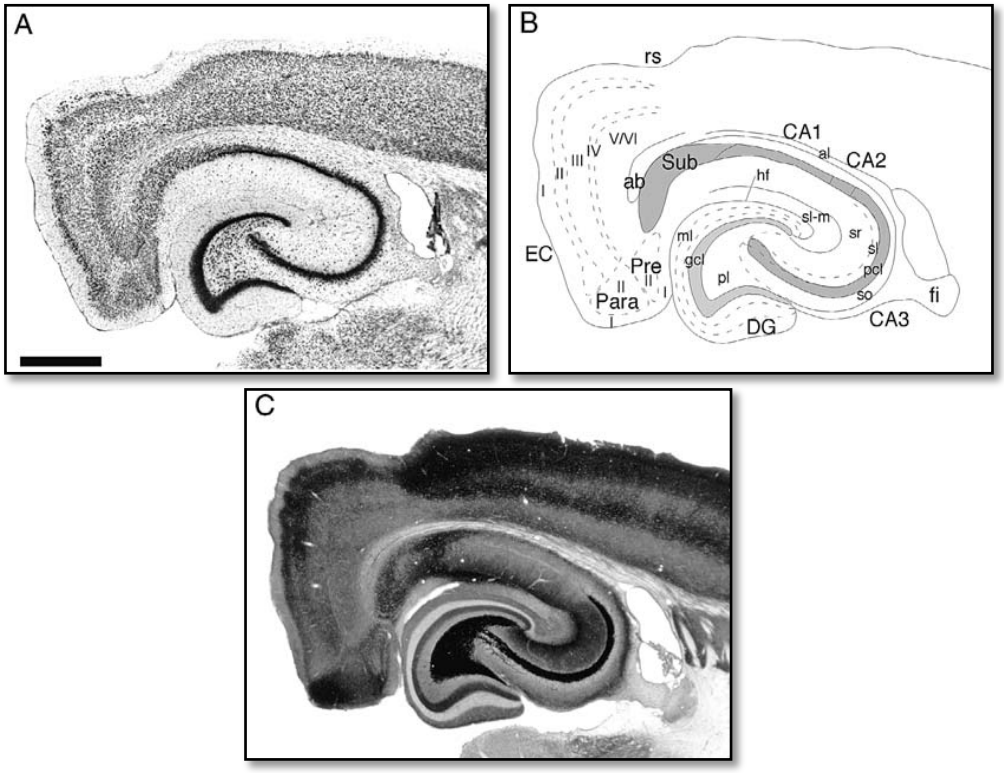

Figure 2.1.1 – Horizontal sectioning of the rat hippocampal formation. A. Nissl-stained section. B. Line drawing showing the various regions, layers, and fiber pathways. C. Timm's sulfide silver-stained section. CA1 - CA1 field of the hippocampus; CA2 - CA2 field of the hippocampus; CA3 - CA3 field of the hippocampus; DG - dentate gyrus; EC - entorhinal cortex. Adapted from Andersen *et al*. (2007).

2.2 - Synaptic transmission

Modern neuroscience, as we know it, was born more than a century ago when Santiago Ramón y Cajal found the first scientific results that supported the neuron doctrine. This doctrine consists in the idea that neurons work as the signalization functional unites in the nervous system, communicating with each other's in a precise and specific way (Albright *et al.* 2001). The acceptance of this doctrine represented a radical and definitive paradigm shift to a more emphatic cellular perspective of the brain. Ramón y Cajal pertinent observations led to the development of the principle of

dynamic polarization. According to this principle, electric signalization between neurons is unidirectional; in other words, the signals propagate from the receiving pole of the neurons – the dendrites – to the presynaptic axon terminal (Albright *et al.* 2001). This way, the afferent and efferent elements are associated in the grey matter by unidirectional contact between the neurons (Breathnach, 2005). A few years later, Sherrington found that not all synaptic actions were excitatory and that some of them can be inhibitory (Sherrington, 1932 in Albright *et al.* 2000). Sherington and Eccles discoveries implied that each neuron solves the competition between excitation and inhibition using, at is initial segment, a winner-takes-all strategy. As a result, an elementary aspect of the integrative action of the brain could now be studied at the level of individual cells. One could determine how the summation of inhibition or excitation leads to an integrated, all-or-none output at the initial segment. Although the initial studies were developed in motor neurons, soon it was discovered that this results had a predictive value in all brain neurons (Albright *et al.* 2001).

The initial task in understanding the integrative functions of the brain consisted in twigging signal integration in individual cells. Edgar Adrian and John Langley, two contemporaries of Sherrington, developed methods of single-unit analysis within the central nervous system. With these methods, it was possible to study signalling in individual cell at any part of the nervous system. In the progression of his work, Adrian discovered that virtually all neurons use the same conserved mechanism for signalling within the cell (Albright *et al.* 2001). Therefore, the precise and effective communication between neurons is possible due to the transmission of information through the generation and propagation of an electric signal, acknowledged as the action potential (Adrian, 1957). In all cases, the action potential can be defined as a large, all-or-none, regenerative electric event that propagates, inexorably, from the initial segment of the axon to the presynaptic terminal (Albright *et al.* 2001). They are mediated by voltage-activated Na^{2+} channels, which are initiated following depolarization of the membrane potential to a threshold level (Stuart *et al.* 1997). As all other cells, neurons have differences in potentials across the cell membrane, the inside being more negatively charged than the extracellular side. This different in potential is known as membrane resting potential.

Given the great number of existing neurons in the nervous system, it is vital the presence of appropriate and efficient communication mechanisms between neurons. In 1897, Sherington introduced the term synapse to describe the

unidirectional contacts between neurons (Breathnach, 2005). Synapses can be grouped in two main clusters: electric and chemical synapses. John Langley, a Sherrington contemporary, postulated the first scientific evidence that the majority of the synapses are of chemical nature (Albright *et al.* 2001). In fact, the main mechanism of synaptic transmission found between neurons comprises the neurotransmitter releasing chemical synapse. However, a more simpler and fast mechanism of signalization can be achieved through electric synapses. The main components of these synapses are gap junctions, which allow the ionic current to flow directly between neurons (Connors & Long, 2004). A big majority of electric synapses are appositions of neuronal membranes entitled gap junctions. These electric synapses, also known as electrotonic synapses, allow the communicating between neuronal cells with completely different functional properties than the ones used by chemical synapses (Bennett, 1972). In electric synapses, the transmission of information depends on current flow through the gab junctions that connect the cytoplasm of pre and postsynaptic cells. In the other hand, chemical synapses are separated by an extracellular space, named synaptic cleft. The communication between neurons becomes possible due to the release and action of chemical agents known as neurotransmitters. As a result of neurotransmitters release into the synaptic cleft mediated by presynaptic cells, specific receptors located at the postsynaptic cell are activated. Figure 2.2.2 displays an example of chemical synaptic transmission involving glutamate and AMPA receptors.

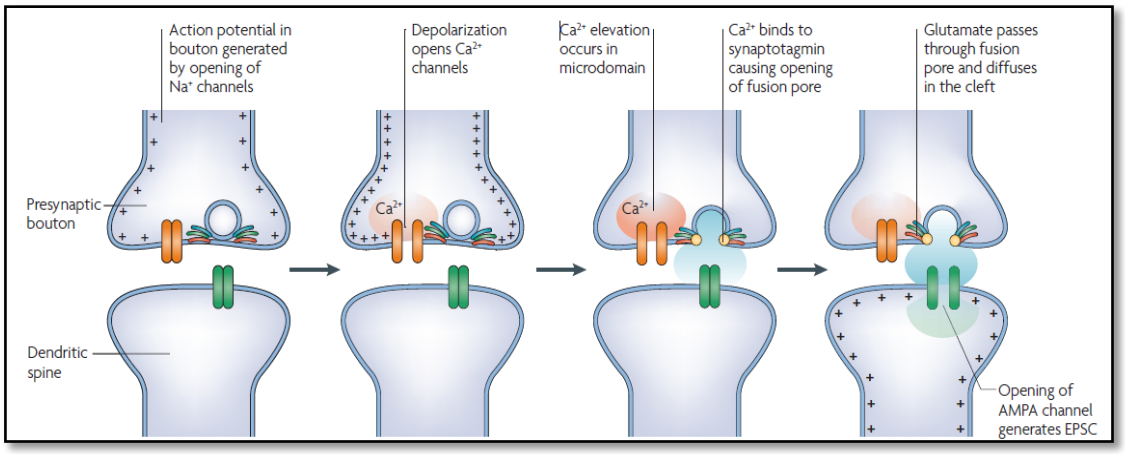

Figure 2.2.2 – Chemical synaptic transmission. These steps can happen during chemical neurotransmission at the neuromuscular junction as well as in central synapses in both vertebrates and invertebrates. This example applies to fast synaptic glutamatergic transmission, but also to others involving different neurotransmitters and corresponding postsynaptic ionotropic receptors. Adapted from Lisman *et al*. 2007.

The receptors located in the postsynaptic cell can belong to one of two receptor classes: ionotropic receptors and metabotropic receptors. Eccles and McGeer described in 1997, for the first time, the two main and different forms of neurotransmission. In the ionotropic transmission, the synaptic receptor located in the postsynaptic cell is an ionic channel, in which the activity is not mediated by voltage (as in the sodium and calcium channels), but instead by a chemical ligand, the neurotransmitter (Albright *et al.* 2000). On the other hand, the metabotropic actions of neurotransmitters mediate the production of second messengers that consequently produce metabolic alterations in the postsynaptic cell (Schoepp *et al.* 1999).

Depending on the type of neurotransmitter that is mainly produced in the presynaptic cell and the type of receptor mainly expressed in the postsyanptic cell, synapses can be characterized as excitatory or inhibitory. Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and mediates its actions via activation of both ionotropic and metabotropic receptors families (Arundine & Tymianski, 2003). There are three different types of ionotropic glutamate receptors, which are named after the agonists that were originally identified as the substances that selectively activate them: **N**-methyl-D-aspartate (NMDA) receptors, **α**-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainato) receptors. Concerning metabotropic glutamate receptors, there are eight different receptor subtypes identified (Kew and Kemp, 2005).

2.3 - NMDA receptors

2.3.1 - NMDA receptor characterization

Understanding the functional mechanisms and the expression of NMDA receptors has captured a lot of attention in the last decades. The intense study and debate about NMDA receptors has provided a great deal of knowledge about its synthesis and cellular trafficking. NMDA receptor molecular and biological proprieties, as well as the changes in their expression during synaptic transmission, evolved to two vastly studied subjects mainly due to the discovery that NMDA receptors are key components in the induction of several forms of synaptic plasticity (Groc *et al.* 2009). Likewise, long lasting changes in synaptic efficiency caused by NMDA receptor activity, as well as the observation that their activity can be

associated to damage caused by ischemia (Rothman & Olney, 1995) and to epilepsy (Rogawski, 1993), were two other important factors that led to the increased interest in studying NMDA receptor activity (Dilmore & Johnson, 1998). NMDA receptors exhibit one unique property that allows them to work as a coincident detector for correlated activity. This integrated activity appears to be required for the refinement of synaptic connections throughout brain development, and for activity-dependent potentiation or depression of synaptic inputs (Zarain-Hezerberg *et al.* 2005).

NMDA (*N*-methyl-D-aspartate) receptors are inotropic glutamate receptors (Bakkar *et al.* 2011). When compared with other glutamate receptors, their main characteristics are voltage-dependent activity, high calcium permeability and slow activation-deactivation kinetics (Doherty & Sladek, 2011). NMDA receptors can be found in the synapse, in extrasynaptic locations and presynaptically (Groc *et al.* 2009). In immature hippocampal neurons, extrasynaptic NMDA receptors represent three-quarters of all the receptors contained in these cells. Although there is an increase in the amount of synaptic NMDA receptors during brain development, a substantial amount of NMDA receptors preserve their extrasynaptic localization throughout the adult life (Hardingham & Bading, 2010). In the synapse, NMDA receptors can be found in the postsynaptic density, where they are structurally organized and spatially restricted in a large signalling macromolecular complex of synaptic scaffolding and adaptor proteins, such as the PSD-95 (postsynaptic density protein 95) family of proteins, or MAGUKS (membrane-associated guanylate kinases). Besides regulating synaptic targeting of glutamate receptors and plasticity in excitatory synapses, these proteins are responsible to physically connect NMDA receptors to kinases, phosphatases and other downstream signalling proteins, as well as to metabotropic glutamate receptors (mGluRs) (Lau & Zukin, 2007).

2.3.2 - NMDA receptor structure

NMDA receptors are heteromeric molecules formed by three subunits, namely subunits GluN1, GluN2 and GluN3. Each one of these subunits contains several variants: single GluN1 subunits with eight splice variants, that are formed through different combinations in the GRIN1 gene, four GluN2 subunits and two GluN3 subunits (Groc *et al.* 2009). Molecular diversity in NMDA receptor occurs due to alternate RNA splicing in GluN1 subunit (Lau & Zukin, 2007). Alternative splicing of

the same gene creates distinct isoforms responsible for NMDA receptor composition variation (for review see Doherty & Sladek, 2011; Zarain-Herzberg *et al.* 2005). Unlike GluN1 subunit that is constitutively expressed in NMDA receptor, GluN2 subunit expression is largely regulated during brain development (Tabish & Ticku, 2003). Through molecular screenings of NMDA receptor subunit composition, four GluN2 subunits, each encoded by a different gene, have been identified and described (Ishii *et al.* 1992). Besides these four GluN2 subunit-expressing genes, in mammals there is a fifth gene that encodes the GluN1 subunit, primarily identified by Moriyoshi in 1991 (Cox *et al.* 2005). A sixth unique GluN3 subunit receptor has been also identified and derives from a different and independent gene. The physiological signature of the receptor is determined by the co-association between a GluN1 subunit and a GluN2 (A, B, C or D), or between a GluN1 subunit and a GluN3A. It is not clearly understood how this co-assembles between subunits works, but it is believed that it reflects the genetic expression of the encoding genes in specific brain regions (for review see Goebel & Poosch, 1999).

In CA1 hippocampal region, NMDA receptors are tetraheteromers, typically composed of two mandatory glycine-binding GluN1 subunits and two modulatory glutamate-biding GluN2 subunits (Doherty & Sladek, 2011). While GluN1 subunit is essential to the formation of a functional NMDA receptor, different combinations between GluN2 and GluN3 subunit types determines biophysical and pharmacological properties of NMDA channels (Takahashi, 2005). Therefore, GluN2 and GluN3 subunits award distinct properties to each receptor, such as sensitivity to glutamate, peak open probability and response kinetics (Zarain-Herzberg *et al.* 2005). NMDA receptor subunit composition can affect the development, maintenance and stability of synapses through various potential mechanisms (Tovar & Westbrook, 1999). As shown in figure 2.3.2.1 displayed below, in the synapse, NMDA receptors are connected to a large multi-protein complex through GluN1 and GluN2 cytoplasmic C-terminal (Collins *et al.* 2006). This complex facilitates NMDA receptor localization in specific areas, simplifying receptor connexions to a variety of signalling molecules that mediate many of the effects induced by NMDA receptor activity (Hardingham, 2009).

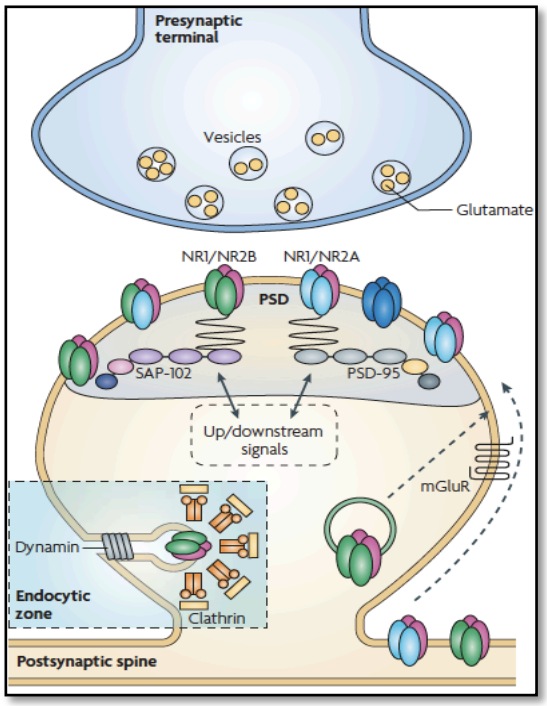

Figure 2.3.2.1 – NMDAR macromolecular signalling complex at excitatory synapses. Synaptic NMDARs are localized in the postsynaptic density PSD (grey area), where they are structurally organized and spatially restricted in a large macromolecular signalling complex comprising scaffolding and adaptor proteins. These proteins physically link the receptors to signalling proteins and to group I mGluRs, which localize to the perisynaptic region. At mature synapses NMDARs are predominantly composed of GluN1/GluN2A or GluN1/GluN2A/GluN2B assemblies. The scaffolding proteins link NMDARs directly or indirectly to a number of signalling proteins, such as protein kinase A (PKA) and PKC, which are thought to be important in the regulation of NMDAR number and function at synaptic sites (upstream/downstream signalling molecules). Adapted from Lau & Zukin, 2007.

2.3.3 - NMDA receptor activity

The primary function of ionotropic glutamate receptors is to respond to presynaptically released transmitter by generating depolarization and excitatory postsynaptic potentials (MacDonald *et al.* 2007). In order to be active, NMDA receptors need to undergo membrane depolarization and the ligand binding needs to be present (Doherty & Sladek, 2011). As a result of their activity, rapid and high-resolution excitatory signals are transmitted from the presynaptic neuron to the postsynaptic one. Temporal and spatial summation of excitatory postsynaptic potentials in a single postsynaptic neuron provides convergence and integration of a large diversity of excitatory inputs from many presynaptic neurons (MacDonald *et al* 2007). NMDA receptor activity is essential for the regulation of almost all excitatory

activity in the brain (Doherty & Sladek, 2011). Almost every single excitatory glutamatergic synapse contains NMDA and AMPA receptors. However, excitatory postsynaptic potentials are almost entirely generated by the current flow across AMPA receptors, due to the NMDA receptor voltage dependence (MacDonald *et al.* 2007). In fact, the present of extracellular Mg^{2+} leads to a fast blockage of NMDA receptor channel, as it is represented in figure 2.3.3.1 displayed below.

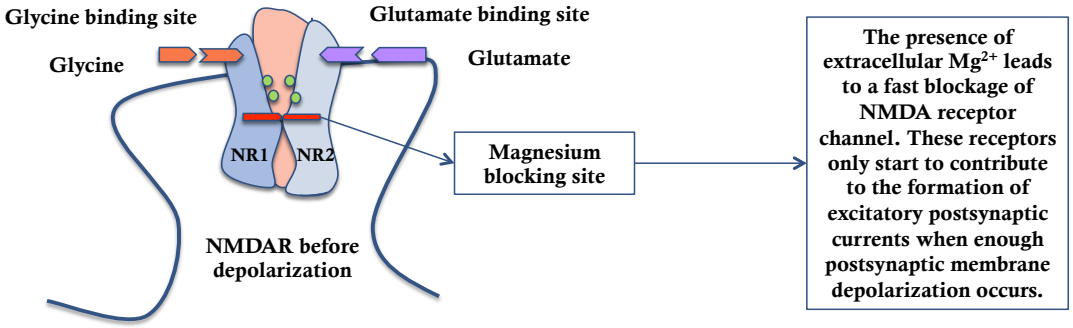

Figure 2.3.3.1 – NMDA receptor at basal condition. In basal activity conditions excitatory postsynaptic potentials are almost entirely generated by AMPA receptors due to NMDA receptor voltage dependence (MacDonald *et al*. 2007).

NMDA receptors only start contributing to the formation of excitatory postsynaptic potentials when occurs enough postsynaptic membrane depolarization. As it can be seen in figure 2.3.3.2, the summation of temporal and spatial excitatory postsynaptic potentials results in the ceasing of the extracellular Mg^{2+} blockage effect and in depolarization of the membrane (McBain & Mayer, 1994).

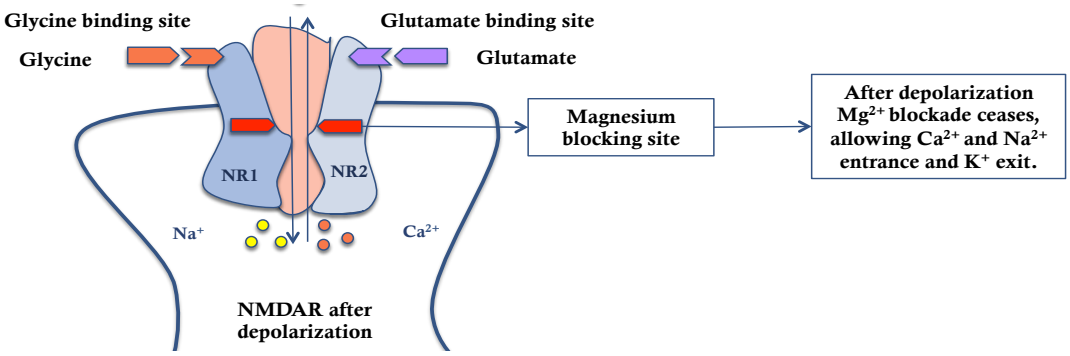

Figure 2.3.3.2 – NMDA receptor after depolarization of the membrane. After the summation of temporal and spatial excitatory postsynaptic potentials the membrane is depolarized and the Mg^{2+} blockage ceases (McBain & Mayer, 1994; McDonald *et al*. 2007)

NMDA receptors are essential to LTP (long term potentiation), an activity dependent increase in synaptic efficiency (Larkman & Jack, 1995) mainly expressed in the hippocampus, which can underlay memory and spatial learning processes (Moriyoshi *et al.*, 1991). This phenomenon was initially described in the seventies, when it was postulated that an evoked response in the dentate area by single test shocks to the afferent perforant pathway, often remained potentiated for a considerable time, even after relatively short periods of stimulation (Bliss & Lomo, 1973). The activity of NMDA receptors results in a significant Ca^{2+} influx increase. As a consequence, NMDA receptor activity is associated with a large variety of intracellular signalling pathways that are dependent on Ca^{2+} influx variations (MacDonald *et al*. 2007). For instance, the role of NMDA receptor in LTP triggering is largely associated to an increase in Ca^{2+} ion concentration inside the cell. This increase occurs when the postsynaptic cell is depolarized due to a large release of glutamate in the synaptic cleft. This way, the extracellular Mg^{2+} dissociates from the coupling location in the receptor, allowing Ca^{2+}, as well as Na^{+} ions, to enter the dendritic spine. The consequent increase in Ca^{2+} ion concentration inside the cell is the critical element to LTP triggering (Malenka & Nicoll, 1999), as it is displayed in figure 2.3.3.3.

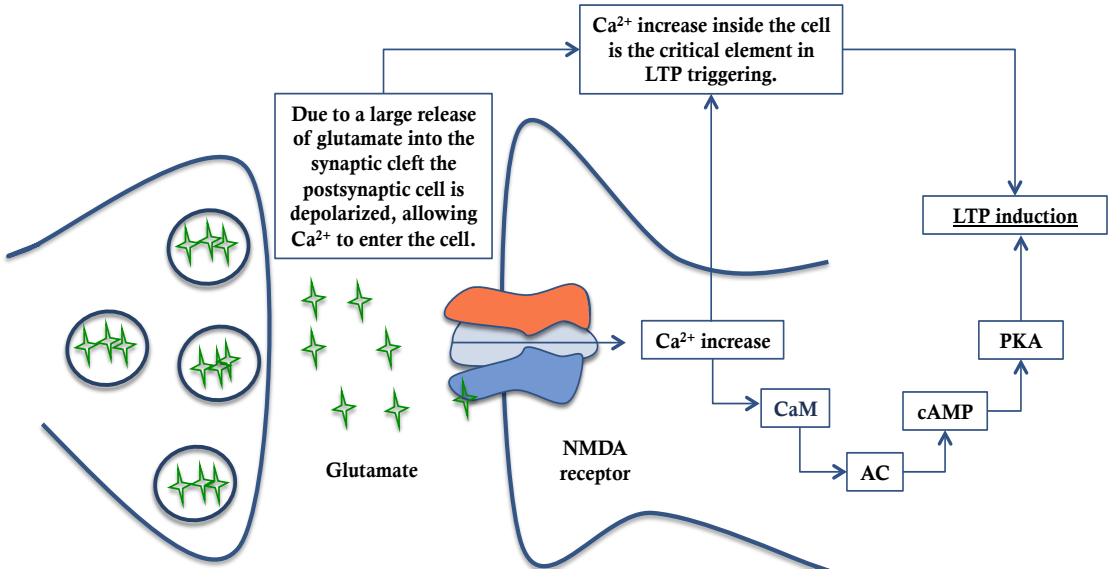

Figure 2.3.3.3 – NMDA receptor at basal condition and after depolarization of the membrane. NMDA receptor activity results in a significant Ca^{2+} influx increase associated with a large variety of intracellular signalling pathways dependent on Ca^{2+} influx variations (Malenka & Nicoll, 1999).

Since their discovery in the seventies and the successful cloning of the receptor accomplished by Moriyoshi in 1991, NMDA receptors gain a role as one of the principal study objects in the attempt to discover the neurobiological mechanisms underlying memory and learning processes. Their study is particularly useful due to four unique NMDA receptor features: the extracellular Mg^{2+} voltage dependent blockage, their permeability to the passage of Ca^{2+}, their slow kinetics and the presence of glycine as a co-agonist that offers another regulatory component that helps in the precise manipulation of these receptors (Ha *et al.* 2006).

2.3.4 - NMDA receptor paradox

The variations in Ca^{2+} concentration inside the postsynaptic cell are the main cause underlying the "NMDA receptor paradox" (Hardingham & Bading, 2010). NMDA receptors are the main responsible agents for the excessive and neurotoxic increase of Ca^{2+} inside the neuronal cell. When the postsynaptic cell is exposed to high levels of glutamate, it can lead to an over activation of NMDA receptors and consequently to a significant increase in Ca^{2+} influx inside the postsynaptic neuron. Both these process can lead to cellular signalling disruption contributing to neuronal cell death (Xu *et al.* 2009). In fact, Ca^{2+} entrance through NMDA receptors is a particular efficient way of causing neuronal cell death, when compared to its entrance through other ionic channels (Hardingham & Bading, 2010). Glutamate excitotoxicity is identified as a key component in many neurodegenerative and neurologic diseases (Xu *et al.* 2009). In chronic neurodegenerative diseases, where the insult is mild, excessive NMDA receptor activity leads to neuronal apoptosis. On the other hand, in cases as stroke that can lead to ischemia, the insult is fulminant and the excessive activity of NMDA receptors can lead to cellular necrosis (Lipton & Nakanishi, 1999).

However, NMDA receptors are also linked to neuronal survival molecular pathways and to synaptic plasticity processes (Xu *et al.* 2009). They play key roles in controlling the morphological and functional plasticity of synapses and contribute to the cellular mechanisms of memory and learning. In pathologies as Alzheimer's disease, synaptic plasticity is severely impaired (Schimtt, 2005). Besides allowing a direct signalization between neurons, NMDA receptors are also important for the correct neuronal development and or the establishing of synaptic contacts (MacDonald *et al.* 2007). Supressing NMDA activity in *in vivo* models significantly

increase cellular apoptosis mechanisms and traumatic lesions in developing neurons (Hardingham & Bading, 2010).

During several years, the amount of Ca^{2+} that enters in the postsynaptic neuron through NMDA receptors was considered as the main and only accountable cause related with the different NMDA activity consequences (Lipton & Nakanishi, 1999). Therefore, moderated levels of NMDA activity were considered healthy for the neurons, while NMDA receptors over activation could lead to an overcharge of intracellular Ca^{2+} harmful to neuron health. According to this model, neuron responses to NMDA receptor activity can be described as a bell-shaped curve, where both a high and a low amount of receptor activity can be harmful for neuronal cell health (Hardingham & Bading, 2010).

Due to more recent discoveries, this later model of cellular behaviour responses to the changes in NMDA receptor activity has been adjusted. According to the new perspective of this paradox, synaptic NMDA receptor location is the main responsible cause for the different cellular consequences following NMDA receptor activity (Hardingham & Bading, 2010). Recent investigations have described several examples of opposing effects in neuronal signalling pathways and cellular survival concerning synaptic and extrasynaptic NMDA receptors activity (Stark & Bazan, 2011). In fact, the duality in NMDA receptor mediated processes varies accordingly to their synaptic or extrasynaptic localization. Synaptic NMDA receptors are related with the induction of signalling survival processes, while extrasynaptic NMDA receptors are associated with Ca^{2+} disruption and neuronal cell death (Stark & Bazan, 2011). Therefore, cellular consequences of NMDA receptor activity are directly associated to the receptor localization and not only with the amount of stimulation and Ca^{2+} entrance as previously mentioned (Hardingham *et al*. 2002). These data can be better described through an "x" shaped graph (see figure 2.3.4.1 below). The ascendant curve of the "x" represents the upsurge in neuroprotection associated with synaptic NMDA receptor activity increase. This ascendant curve is superposed to a descendent one, that illustrates the progressive decrease in neuroprotection related with the increase in extrasynaptic NMDA receptor activity (Hardingham & Bading, 2010). Accordingly to this perspective, it is not the Ca^{2+} overcharge *per se* the sole determinant of neurotoxicity but rather the Ca^{2+} influx through NMDA receptors localized outside the synapse that are responsible for the prejudicial cellular consequences. The influx of Ca^{2+} through synaptic NMDA receptors is well tolerated

by the neuronal hippocampal cells. Furthermore, it represents the required mechanism for the activation of genomic processes that can give rise to neuronal cells that are more resistant to apoptosis and oxidative processes (Hardingham *et al.* 2002).

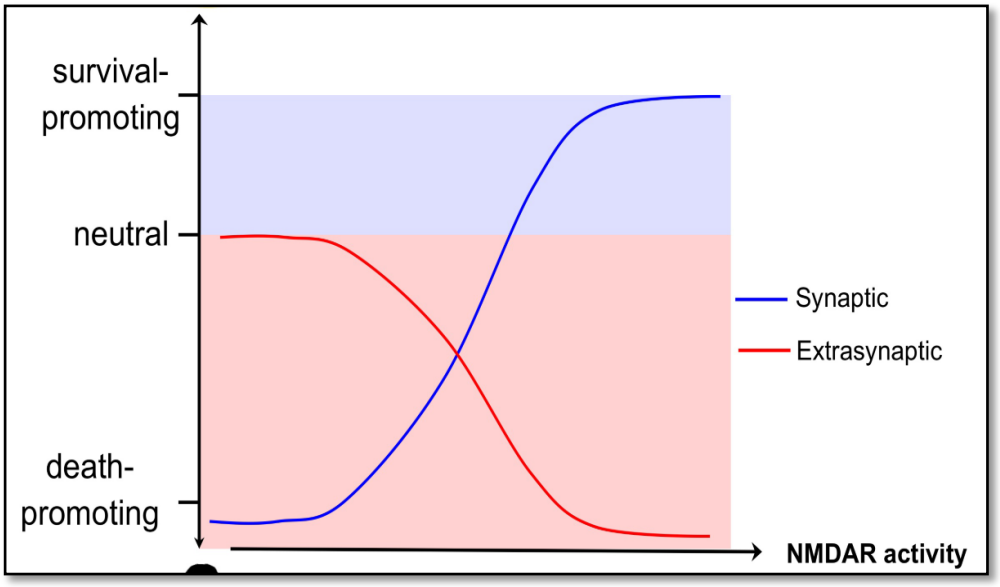

Figure 2.3.4.1 – Synaptic vs. Extrasynaptic NMDA receptor activity. The schematic illustrates the opposing effects of increasing synaptic and extrasynaptic N-methyl-D-aspartate receptor (NMDAR) activity on neuronal survival and resistance to trauma. Hypoactivity of synaptic NMDARs is harmful to neurons. Enhancing synaptic NMDAR activity triggers multiple neuroprotective pathways and this promotes neuronal survival. Low levels of activation of extrasynaptic NMDARs have no effects on neuronal survival but increasing the level of extrasynaptic NMDAR activity activates cell death pathways and exacerbates certain neurodegenerative processes, thus reducing neuronal survival (adapted from Hardingham & Bading, 2010).

Besides spatial receptor location and Ca^{2+} influx intensity and duration, there is a third factor that can influence the consequences of NMDA receptor activity. Subunit receptor composition may play an important role in neurotoxicity or neuronal survival processes (Hardingham, 2009). Excitotoxicity mediated by NMDA receptor activity may be triggered due to GluN2B composing subunits (Stanika *et al.* 2008). There is an ostensive correlation between the presence of GluN2B subunits in NMDA receptor composition, high cytosolic Ca^{2+} levels and susceptibility to neurotoxicity (Cheng *et al.* 1999).

2.4 - Synaptic vs. Extrasynaptic NMDA receptor signalling

2.4.1 - Synaptic NMDAR-dependent neuroprotection

An episode of synaptic NMDA receptor activity can induce neuroprotection for a long time, even after the receptor activity as ceased (Papadia *et al.* 2005). Although the processes and mechanisms through which nuclear calcium-regulated genomic events increase neuronal survival activity are not fully understood (Dick & Bading, 2010), recently some light has been shed over the subject. Figure 2.4.1.1 presented below displays some of the neuroprotective pathways that are activated through synaptic NMDA receptor activity.

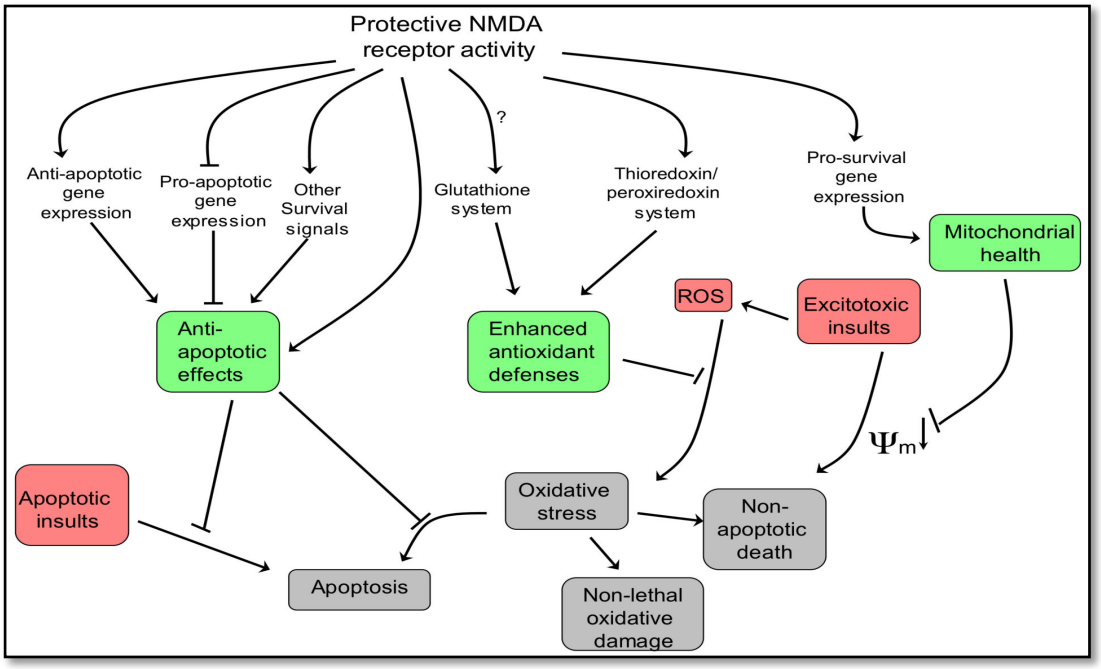

Figure 2.4.1.1 – Neuroprotective pathways activated by synaptic NMDA receptor activity. Adapted from Hardingham & Bading, 2010.

Calcium influx into hippocampal neurons through synaptic NMDA receptors triggers the release of Ca^{2+} ions from intracellular calcium stores. Eventually, these Ca^{2+} ions are transduced to the cellular soma invading the nucleus (Hardingham *et al.* 2001). The spread of activity-induced calcium signals ending in the cell nucleus represents a main route for the communication between the synapse and the cell nucleus (Zhang *et al.* 2009). Nuclear Ca^{2+} is known to be one of the more potent inducers of neuronal gene expression (Hardingham & Bading, 2010). Changes in the

expression of pro-survival genes result in mitochondrial health improvement, the increase of antioxidant defences and the suppression of caspases.

Nuclear calcium-regulated genes are accountable for a neuroprotective shield that neurons build when the synapses are activated (Zhang *et al.* 2009). Among those genes, a very important target of nuclear Ca^{2+} is the nuclear Ca^{2+}/ calmodulin-dependent protein (CaM) IV and the transcription factor cyclic-AMP response element binding protein (Hardingham & Bading, 2010). Synaptic NMDA receptor activity promotes neuroprotection throughout the activation of pro-survival signalling pathways, including cAMP response element-binding protein-dependent (CREB-dependent) gene expression (Soriano *et al*. 2006). CaM kinase IV is an important regulatory protein (Enslen *et al.* 1994) and CREB is the prototypical signal-regulated transcription factor. CREB is a central mediator of neuroprotection being responsible for controlling a number of pro-survival genes (Zhang *et al*. 2005). In hippocampal neurons, CREB-dependent gene expression is linked with long-lasting activity-dependent neuroprotective episodes against apoptotic and excitotoxic insults (Hardingham & Bading, 2010). This acquired and long-lasting form of neuroprotection depends on Ca^{2+} nuclear signalling (Hardingham & Bading, 2010). Through intracellular calcium level manipulations, it has been shown that Ca^{2+} has a main role in CREB activation (Hardingham *et al.* 2001). This neuroprotection arises due to the strengthening of the mitochondria against cellular stress and oxidative or toxic insults (Zhang *et al.* 2009).

Oxidative stress manifestation is related with an imbalance between reactive oxygen species (ROS) and cellular ability to neutralize them *via intrinsic* antioxidant defences. Neurons are a particularly susceptible and vulnerable type of cell concerning oxidant damage due to high formation of reactive oxygen species. The oxidative damage has a cumulative effect throughout aging and is now recognized as a major accountable factor for some neurodegenerative diseases as well as acute cerebrovascular disorders (Papadia *et al.* 2008). In addition to apoptosis-suppression effects, synaptic NMDA receptor activity also displays a key role in enhancing neuronal antioxidant defences (Hardingham & Bading, 2010). Synaptic NMDA receptor activity triggers signalling changes centred on the thioredoxin-peroxiredoxin system that underlie a coordinated program of gene expression changes that ultimately lead to the enhancement of antioxidant effects (Papadia *et al.* 2008).

Ultimately, the expression of these genes will contribute to the preservation of normal neuronal functions and in the improvement of cellular viability when facing aggressive stimuli (Hardingham & Bading, 2010). Also, they can regulate the development and function of individual synapses contributing to synaptogenesis (West *et al.* 2002). In the context of synaptic plasticity, CREB plays a central role in prolonging and consolidating activity dependent changes in synaptic strength (Papadia *et al.* 2005). Another target of nuclear Ca^{2+} - CREB signalling is a gene that encodes the neurotrophin brain-derived neurotrophic factor (BDNF) (Hardingham & Bading, 2010), which is strongly regulated by synaptic NMDA receptor activity (Hardingham *et al.* 2002). Besides showing neuroprotective proprieties, lowered BDNF expression plays an important role in neurodegeneration processes that follow NMDA receptor blockade (Hansen *et al.* 2004). BDNF can rescue neurons from NMDA blockade-induced neuronal cell death (Hardingham & Bading, 2010).

The suppression of neuronal death promoting genes consists in a second mechanism through which synaptic NMDA activity guarantees neuronal cell health (Hardingham & Bading, 2010). As said, long-lasting activity-dependent neuroprotection is triggered by Ca^{2+} entry through synaptic NMDA receptors, requiring the propagation of calcium signals into the nucleus and initiation or repression of gene transcription (Lau & Bading, 2009). Transcriptional suppression of core components of the intrinsic apoptosis cascade is regulated by nuclear Ca^{2+} signalling (Hardingham & Bading, 2010). Generation of neuronal apoptosis can occur through up-regulation of BH3-only genes such as *Puma* (Léveillé *et al.* 2010). It is known that synaptic NMDA receptor activity can supress the expression of this specific gene, both *in vitro* and *in vivo*. Similarly to the activation of CREB, suppression of *Puma* is also specifically induced by synaptic NMDA receptor activity. Ultimately, the suppression of these genes expression results in apoptosis inhibition, increasing neuronal viability (Hardingham & Bading, 2010). Also, the tumour suppression gene, *trp53* and two of its targets are regulated by synaptic NMDA receptor activity. Suppression of *trp53* acts mutually with nuclear activity and nuclear calcium-induced neuroprotective genes and jointly promotes neuronal surviving through inhibition of mitochondria permeability transition (Lau & Bading, 2010). Mitochondrial health improvement represents a key factor in neuroprotective synaptic NMDA receptor activity mediated pro-survival signalling (Lau & Bading, 2010). Synaptic NMDA receptor activity also subdues the expression of important

pro-death transcription factors such as the FOXO family of Forkhead transcription factors (Hardingham & Bading, 2010). FOXO dependent cellular responses include gluconeogenesis, neuropeptide secretion, atrophy, autophagy, apoptosis, cell cycle arrest and oxidative stress (Salih & Brunet, 2008). The inhibition of FOXO nuclear import is long lasting (Al-Mubarak *et al.* 2009), which means that it can be sustained for a considerable period of time, even after synaptic NMDA activity has ceased (Hardingham & Bading. 2010).

Also, activation of synaptic or extrasynaptic NMDA receptors that contain in their composition GluN2A subunits is correlated with neuronal survival promoting mechanisms. Furthermore, these receptors can function as a neuroprotective agent against neuronal damage provoked by NMDA receptors or other brain components (Liu *et al.* 2007).

2.4.2 - Extrasynaptic pro-death signalling pathways

Neuronal death dependent from NMDA receptor activity can be mediated by a variety of signalling pathways that are related to the cell type, the developmental stage or the intensity of the excitotoxic insult. Compared to synaptic NMDA receptor activity, extrasynaptic NMDA receptor activity has been shown to be highly associated with certain pro-death signalling pathways (Hardingham & Bading, 2010).

In direct contrast to what was referred just before, an episode of extrasynaptic NMDA receptors activity promotes CREB inactivation and excitotoxicity events such as mitochondrial depolarization in hippocampal neurons (Soriano *et al.* 2006). Moreover, CREB dephosphorylating induced by extrasynaptic NMDA receptor activity superimposes CREB activating signals (Sala *et al.* 2000). The extrasynaptic complexes can act as an antagonist towards nuclear signalling pathways to CREB, blocking the induction of BDNF expression resulting in mitochondrial dysfunction and neuronal cell death (Hardingham *et al.* 2002).

Investigation in the last two decades has proved that calcium influx into neurons through voltage-gated or ligand-gated ion channels does not imply necessarily a unique and pre-ordained response from the cell. In fact, neurons are wired in complex ways, allowing them to respond differently to calcium signals with distinct characteristics. These crucial parameters include a variety of calcium signal features such as magnitude, spatial properties, oscillatory frequency and the channel

through which the calcium initially flows into the neuron (Hardingham & Bading, 2002).

Different channels are associated with different signalling molecules or adaptors that can be specifically activated when calcium signals flows through those specific channels. An overwhelming example of calcium entry site-specific differences is the regulation of transcription mediated by the CREB-binding protein CREB (Hardingham & Bading, 2002). Synaptic and extrasynaptic NMDA receptors are coupled to survival and cell death pathways respectively. These differences are explained mainly due to their opposing effects on the cAMP response element binding protein (CREB) (Bengston *et al.* 2008). CREB regulates the expression of many proteins, including neurotrophic factor BDNF. This protein is highly studied in neuronal plasticity field of investigation due to its engagement in both early and late phases of LTP. Also, a polymorphism of the gene that encodes BDNF is linked to impaired episodic memory in humans, underlying the possible relevance for BDNF in cognitive functions (Vanhoutte & Bading, 2003). In experiments using bath glutamate it was shown that this condition fails to increase BDNF expression due to shut-off of CREB activity, triggered by extrasynaptic NMDA receptors activated by glutamate (Sala *et al.* 2000). CREB shut-off is induced by a rapid dephosphorylation of its active-site amino acid, serine 133 (Vanhoutte & Bading, 2003). The duration over which serine 133 remains phosphorylated affects considerably the degree of CREB-dependent transcription. The dephosphorylation of CREB on serine 133 blocks CREB-dependent reporter gene expression that is initiated by calcium influx through either L-type channels or synaptic NMDA receptor activity. This way, the observation that bath glutamate rarely promotes CREB activation being, on the other hand, highly associated with CREB dephosphorylation can be explained by the dominance of extrasynaptic NMDA receptor activity over synaptic NMDA receptors signalling (Hardingham & Bading, 2002).

Synaptic and extrasynaptic NMDA receptor signalling triggers completely different processes. In fact, synaptic and extrasynaptic NMDA receptors stimulate or inhibit the expression or suppression of the same genes or processes in an opposing way. Figure 2.4.2.1 summarizes the different signalling pathways induce by synaptic and extrasynaptic NMDA receptor activity.

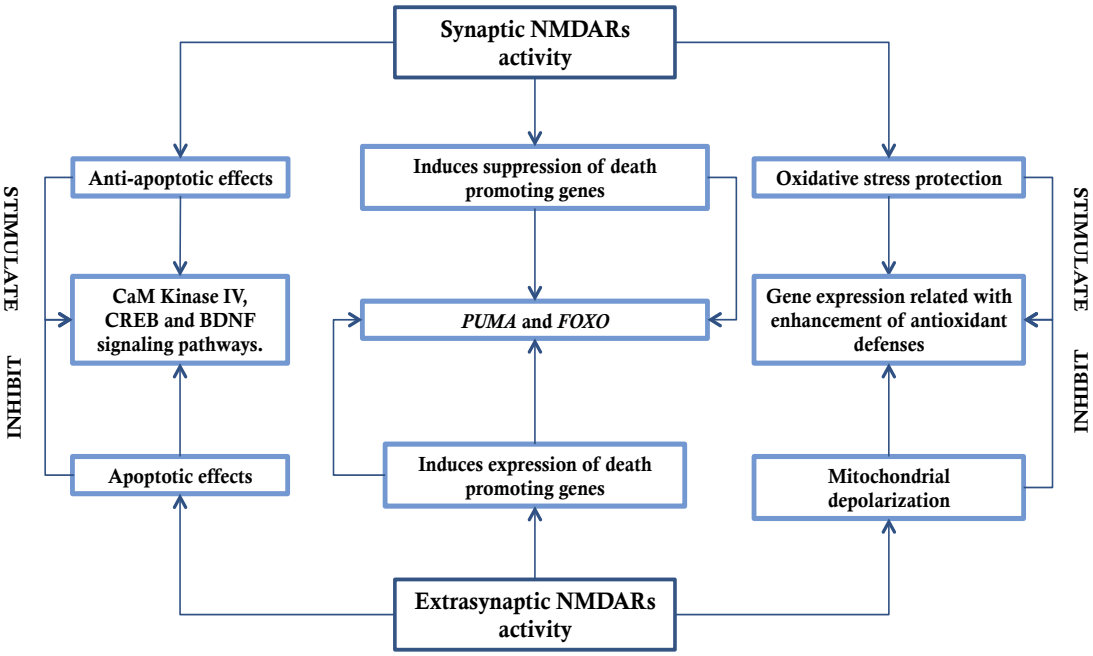

Figure 2.4.2.1 – Summarizing the different signalling pathways induced by synaptic vs. extrasynaptic NMDA receptor activity. Based on Hardingham & Bading, 2010 review.

2.5 - Neuromodulation

Traditionally, the communication between neurons within a neuronal circuitry has been thought to implicate classical neurotransmission. In this classic form of communication, one neuron either inhibits or excites the other neuron with which it synapses on (Katz & Frost, 1996). Neurons communicate directly with each other by chemical synaptic transmission across synapses. Generally, neurotransmission acts through ligand-gated ion channels and the neurotransmitters involved in synaptic transmission are though to mediate brief postsynaptic responses that are spatially restricted. However, some of these agents can also modulate pre and postsynaptic responses without necessarily evoking postsynaptic potentials. Neuromodulation differs from neurotransmission in that it does not necessarily implicate excitation or inhibition from one neuron to another (Katz & Frost, 1996). Neuromodulators act through intracellular second messenger cascades that origin slower, spatially diffused and long-lasting responses (Gelinas & Nguyen, 2007), which alter the cellular or synaptic proprieties of neurons (Katz & Frost, 1996). Anatomic wired clusters of neurons in the central nervous system can be reconfigured by neuromodulators. These neuron clusters are responsible for the formation of networks that will produce

specific rhythmical patters of activity (Pflüger, 1999). In figure 2.5.1 are represented tow second messenger system that are relevant to this work.

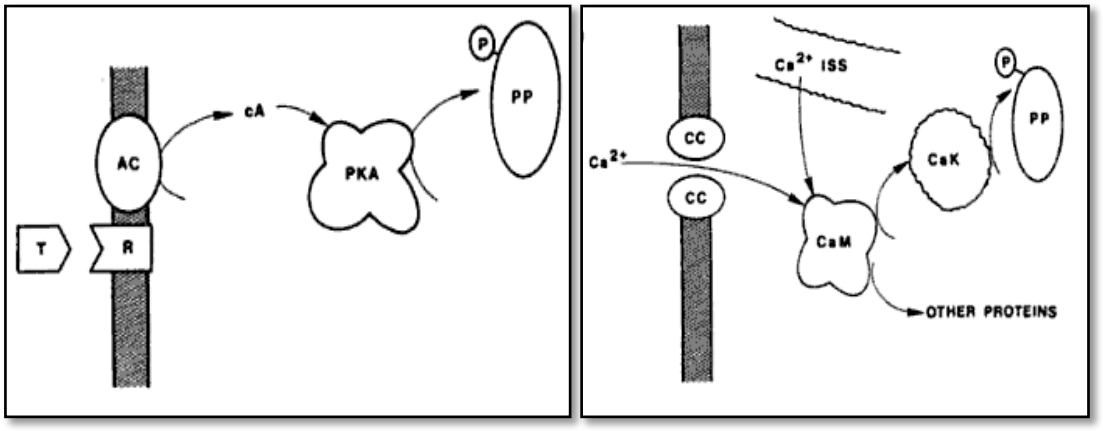

Figure 2.5.1 – Simplified reaction schemes of two messenger systems of interest to this work. In the left: The adenylate cyclase/cyclic AMP-dependent protein kinase system. Adenosine receptors are known to couple with G proteins that, depending of the adenosine subtype receptor, either inhibit (van Calker *et al*. 1979) or stimulate (Corvol *et al*. 2001) the expression of adenylate cyclase. In the right: Intracellular calcium ions as second level messengers. NMDA receptor activity is particularly linked with Ca^{2+} influx inside the postsynaptic cell being associated with a large variety of intracellular signalling pathways dependent on Ca^{2+} influx variations, as LTP induction (Malenka & Nicoll, 1999). Drawings adapted from Kaczmarek & Levitan, 1987.

The concept of neuromodulation was initially recognized as a mechanism where an endogenous substance, which could be released from the pre or postsynaptic component, influences the release (presynaptic modulation) or the action (postsynaptic modulation) of the neurotransmitter (Sebastião & Ribeiro, 2009). Described in a restricted way, neuromodulation can be defined as the neuronal ability to change electrical proprieties in response to intracellular biochemical changes resulting from synaptic or hormonal stimuli (Kaczmarek & Levitan, 1987). Figure 2.5.1 displays some of the changes that the neuromodulators can produce upon the electrical properties of neurons.

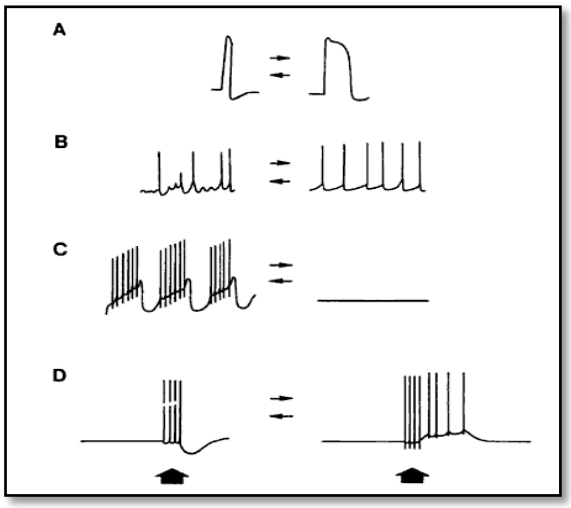

Figure 2.5.2 – Some of the changes that can be observed in the electrical properties of neurons. A) Alterations in the shape of action potentials; B) Changes in frequency and pattern of firing; c) Inhibition and onset of bursting; D) Changes in response to stimulation (arrow). Taken from Kaczmarek & Levitan, 1987.

Neuromodulators can modify neural processing in many different ways regulating various neurophysiological processes (Stern *et al.* 2007). Therefore, they can affect the integrative proprieties of neurons, by altering the way through which they respond to synaptic drive, modify the strengths of the synaptic interconnections (Sillar *et al.* 2002) and reshape the plasticity of those synapses (Dayan, 2012). By changing the properties of neurons, neuromodulators can reshape the output of a circuit (Katz & Frost, 1996) and reconfigure the network, producing different output patterns, hence, making the network multifunctional (Pflüger, 1999). By doing so, neuromodulation allows the central nervous system to adapt its control of psysiological functions to a continually changing environment (Kaczmarek & Levitan, 1987). Biochemical changes that lead to modifications in the electrical features of a neuron may be triggered in one of three ways: by the action of neurotransmitters at specific synaptic junctions, by neurotransmitters secreted at less specialized release sites at a distance from the target neuron and finally, through the action of hormones (Kaczmarek & Levitan, 1987).

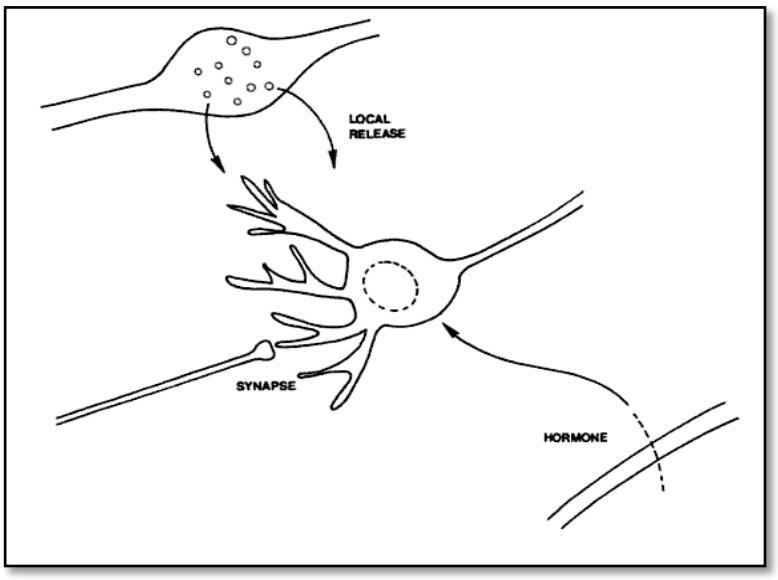

Figure 2.5.3 – Stimuli that can modulate neuronal activity. 1) Synaptic release of neurotransmitter; 2) Local release of agents from neuronal processes not in direct contact with the target cell; 3) Actions of hormones released from other tissues. Taken from Kaczmarek & Levitan, 1987.

Previously, neuronal interaction diagrams rarely incorporated neuromodulation, including only fast synaptic contacts. First, neuromodulation was usually viewed as being originated from a source extrinsic to the circuit and therefore not a part of the circuit itself. Secondly, even when neuromodulatory interactions are intrinsic to the neuronal circuit, these interactions can easily be overlooked because of their slow time course or because their effects are contingent upon the activity of the neurons in which they act (Katz, 1995). Yet, it is now establish that both intrinsic and extrinsic neuromodulation are important for neuronal circuits control. It is important to distinguish intrinsic from extrinsic neuromodulation to fully comprehend neuromodulatory functions in the operation of neuronal circuits. In extrinsic neuromodulation, a circuit receives modulatory inputs from neurohormones or other areas of the nervous system. Due to its location outside the circuits that it affects, a single extrinsic source can modulate different circuits simultaneously. Additionally, a single circuit can receive extrinsic neuromodulatory inputs from several different sources, allowing the circuit do be differentially modulated under different conditions (Katz, 1995). Intrinsic modulation it is not optional, rather mandatory. Moreover, by contrast with extrinsic neuromodulation, within intrinsic neuromodulation the neuromodulatory substances are released by neurons that are intrinsic to a circuit, being able to affect other neurons and synapses in the same circuit (Katz & Frost,

1996). Additionally, the amount of neuromodulatory agents release is controlled by activity within the circuit itself, not by the activity of a distant region or structure in the nervous system (Katz, 1995). Consequently, intrinsic neuromodulation occurs whenever the circuit is activated and the level of intrinsic neuromodulatory action reflects the intensity of the activity in the circuit itself (Katz & Frost, 1996). Figure 2.5.4 presented below displays the main differences between both types of neuromodulation.

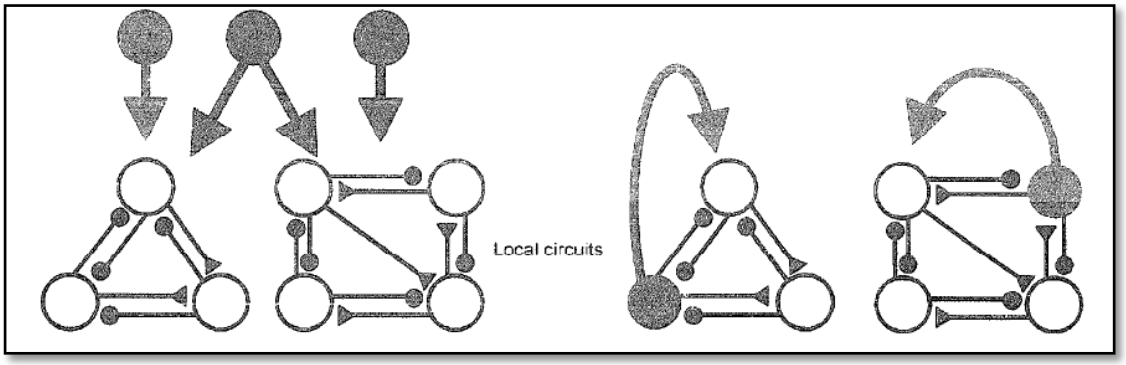

Figure 2.5.4 – Extrinsic and intrinsic neuromodulation. Schematics on the left represent extrinsic neuromodulation originated from neurons outside the local circuit. Different neuromodulatory neurons can be active at different times and in different behavioural contexts. Also, a single neuromodulatory neuron can elicit neuromodulatory effects in many different circuits simultaneously. On the other hand, the schematics on the right panel of the figure represent intrinsic neuromodulation that arises from the activity of neurons within a circuit itself. These neuromodulatory effects are always present whenever the circuit is active. Adapted from Katz & Frost, 1996.

2.5.1 - Adenosine as a neuromodulator

Among the diversity of neuromodulators existent in the nervous system, adenosine and ATP are key fine-tuners for three main reasons. First, they are among the most significant components in communication processes between neurons and glia cells. Secondly, adenosine and ATP can affect the release and action of several neurotransmitters and neuromodulators. Finally, adenosine and ATP can be release by almost all cells in the nervous system (Sebastião & Ribeiro, 2009). Purines and purine nucleotides are vital components of all living cells. ATP is used as an energy source in virtually all cellular activity and adenine is a component of nucleic acids. Due to their ubiquitous nature, purines are also a very important component for both

intracellular and extracellular signalling (Dunwiddie & Masino, 2001). While ATP can work as a neurotransmitter in some brain regions (Burnstock, 2007), adenosine is neither stored nor released as a classic neurotransmitter since it does not accumulate in synaptic vesicles. Adenosine is directly released from the cytoplasm to the extracellular space through a nucleoside transporter. Since it is not exocytotically released, adenosine acts as extracellular signalling molecule that modulates synaptic transmission without being a neurotransmitter (Sebastião & Ribeiro, 2009), just as it does in other excitable tissues, such as the heart, where its actions were first described (Drury & Szent-Györgyi, 1929). Many adenosine actions both inhibit and reduce the activity of excitable tissues or upsurge the delivery of metabolic substrates, helping coupling the energy expenditure to the energy supply (Dunwiddie & Masino, 2001).

Adenosine acts as a potent neuromodulator in the central nervous system modulating synaptic activity (Jenner *et al.* 2009) and modulates neuronal activity through G-protein-coupled mechanisms that lead to changes in second-messenger levels and modulation of ion channels. Adenosine modulatory effects can occur presynaptically, by inhibiting or facilitating transmitter release, postsynaptically, through modifications in other neurotransmitters actions, and nonsynpatically by hyperpolarizing or depolarizing neurons (Sebastião & Ribeiro, 2009). Adenosine concentration can upsurge either through an increase in intracellular formation or through the increase in adenosine nucleotides release followed by their rapid degradation. Increased intracellular adenosine formation results mainly from an imbalance between energy supply and activity demands (increases in cellular activity or hypoxia episodes, for instance) that lead to degradation of adenosine triphosphate (ATP) and in an increase in adenosine monophosphate that is easily broken down to adenosine (Jenner *et al.* 2009). Biochemical studies suggest that adenosine nucleotides are a potential source of extracellular adenosine formation, mainly through ATP molecules that are released as a transmitter or from damaged cells (Dunwiddie *et al.* 1997).

2.5.2 – Adenosine receptors

The biological effects of adenosine are mediated by four distinct subtypes of G-protein-coupled cell surface receptors (Tsutsui *et al.* 2004). Adenosine subtype receptors are found throughout the brain and are implicated in diverse neurological

functions and pathologies (Lusardi, 2009). A_1, A_{2A}, A_{2B} and A_3 receptors (A_1Rs, A_{2A}Rs, A_{2B}Rs and A_3Rs) are the four different adenosine receptor subtypes that have already been successfully cloned and pharmacologically characterized in several species (Wirkner *et al.* 2004). Adenosine receptor expression throughout the brain is region-specific (Ribeiro, *et* al. 2002) and varies through brain development (e.g. Shaw *et al*. 1984). A_1 and A_3 adenosine receptors are respectively coupled do Gi and Go, proteins known to inhibit the expression of adenylate cyclase (van Calker *et al*. 1979), while A_{2A} and A_{2B} receptors are coupled to Gs proteins, known to stimulate the expression of adenylate cyclase and to raise intracellular levels of cAMP (Corvol *et al*. 2001). All adenosine receptor subtypes are implicated in the regulation of immunomodulatory molecules synthesis and release (Tsutsui *et al.* 2004). Importantly, while A_1 and A_{2A} subtype receptors display high affinity for adenosine and are excepted to exert important regulatory functions in physiological conditions, A_{2B}Rs and A_3Rs are low-affinity receptors most likely to play a role in pathological conditions featuring increased extracellular concentrations (Dunwiddie & Masino, 2001). The higher density of A_1 and A_{2A} subtype receptors in the brain and the modest impact upon brain functions of A_{2B}Rs and A_3Rs pharmacological manipulation, has led to the idea that adenosine influence in brain functions might mostly depend on the actions of A_1Rs and A_{2A}Rs (Gomes *et al*. 2010). A_1Rs and A_{2A}R are vastly expressed in several brain areas (Lusardi, 2009), as it is possible to observe in figure 2.5.2.1 displayed below.

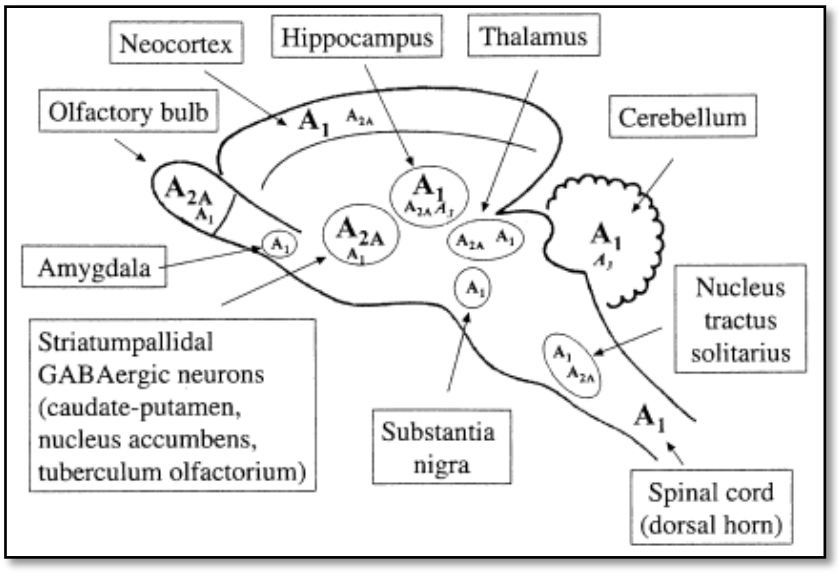

Figure 2.5.1.1 – Distribution of adenosine receptors in the rat brain. Higher levels of expression are indicated by larger alphabets. Adapted from Ribeiro *et al*. 2003

A_1 receptors are highly expressed in the cerebral cortex, while A_{2A} receptors are strongly expresses by straitopallidal GABAergic neurons and are found at much lower levels in other brain regions (see Ribeiro *et al*. 2003), namely the hippocampus. A_1R is the adenosine subtype receptor that can be found in almost all brain regions being associated with K^+ and CA^{2+} channel activation, both related to neuronal activity inhibition (Dunwiddie & Masino, 2001). On the other hand, A_{2A} receptors are expressed in high levels only in some restricted brain regions, namely the striate and other basal ganglia structures (Haas & Selbach, 2000). Figure 2.5.2.2 shows the affinity of each subtype of adenosine receptor and the G-protein protein to which each one of them couples. It is possible to see that both A_1 and A_{2A} receptors display high affinities when compared with A_{2B} and A_3 receptors. Also, it demonstrates the main effects that result from the coupling of adenosine receptors to the specific G-proteins. Adenosine A_{2A} receptors are usually coupled to Gs proteins that trigger cAMP formation and lead to PKA activation (Fredholm *et al*. 2001). On the contrary, A_1Rs are mainly associated to Gi proteins that decrease cAMP formation. Increases in cAMP formation and PKA activation are important to synaptic plasticity processes, including LTP indcution (Malenka & Nicholl, 1999).

	A_1	A_{2A}	A_{2B}	A_3
Adenosine Affinity	~70 nM	~ 150 nM	~ 5100 nM	~ 6500 nM
G-protein coupling	$G_{i/o}$	G_s	G_s G_q	$G_{i/o}$ G_q
Effects of G-protein coupling	↓ cAMP ↑ IP_3 ↑ K^+ ↓ Ca^{2+} currents ↑ MAPK	↑ cAMP ↑ MAPK	↑ cAMP ↑ IP_3 ↑/↓ MAPK	↓ cAMP ↑ IP_3 ↑ MAPK

Figure 2.5.2.2 – Adenosine receptors can couple to several G proteins. Adapted from Dunwiddie & Masino, 2001 and Rees *et al*. 2003.

Adenosine receptors can also influence the action of other neuromodulators and ionotropic glutamate receptors such as NMDA receptors as well as metabotropic receptors (see Sebastião & Ribeiro, 2000).

2.5.3 – A_1Rs and A_{2A}Rs modulatory actions upon NMDA receptor activity

Adenosine is capable of both inhibit and excite neurotransmission in the central nervous system (Fredholm & Dunwiddie, 1988). Adenosine ability to selectively depress excitatory synaptic transmission is the most evident effect of the adenosinergic modulation upon synaptic transmission and the one that was primary studied and characterized in neuronal circuits (Proctor & Dunwiddie, 1987; Thompson *et al.* 1992). This effect can occur due to pre and post-synaptic mechanisms.

Adenosine application was early identified as capable of reducing transmitter release and to depress synaptic potentials (Thompson *et al.* 1992). In fact, adenosine A_1 receptors are linked to inhibition in the release of virtually every classic neurotransmitter including, among others, glutamate, GABA (γ-Aminobutiric Acid), acetylcholine and dopamine. However, the most prominent adenosine inhibitory actions are generally exhibited at glutamatergic excitatory systems. In some cases, adenosine can completely block synaptic transmission upon excitatory glutamatergic transmission.

Activation of adenosine A_1 receptors shows the ability to induce neuroprotective actions in ischemia models as well as neuromodulatory effects on synaptic transmission through inhibition of neurotransmitter release (Barrie & Nicholls, 1993) and by reducing long-term changes in synaptic efficiency (de Mendonça & Ribeiro, 1997). The mechanism of inhibitory modulation of transmitter release has been vastly studied and appears to reflect a G-protein-coupled inhibition of Ca^{2+} channels in nerve endings (Dunwiddie & Masino, 2001). More recently, the low release probability of excitatory synapses was linked with a tonic presynaptic activation of A_1 receptors mediated by ambient adenosine. Indeed, the removal of extracellular adenosine or the inactivation of A_1 presynaptic receptors with antagonists drastically augments synaptic transmission. Additionally, tonic presynaptic activation of A_1 receptors also impairs LTP and NMDA receptor-dependent forms of plasticity (Moore *et al*. 2003). The inhibitory action of adenosine A_1 receptors upon pre-synaptic glutamate release is schematized in figure 2.5.3.1 displayed below.

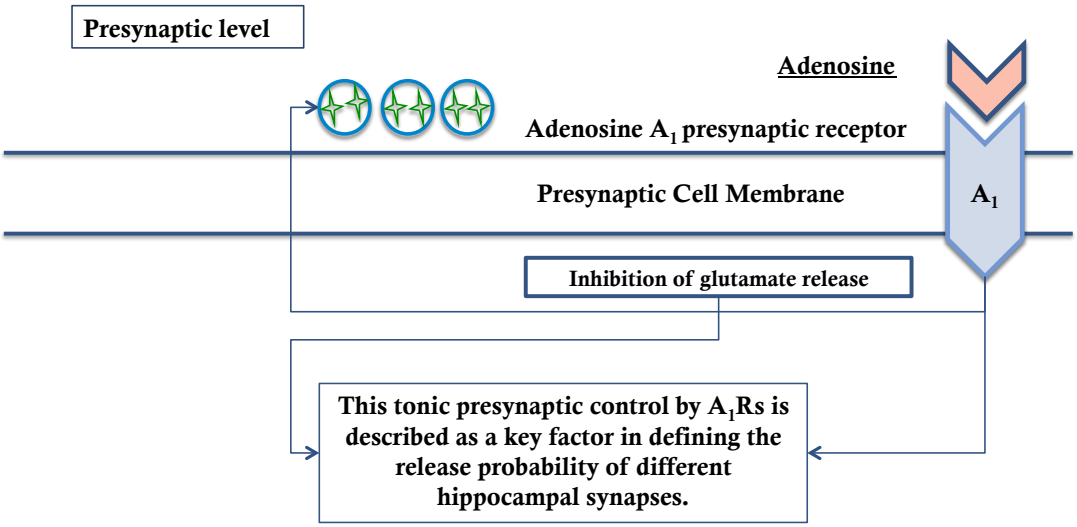

Figure 2.5.3.1 – Presynaptic modulatory action of adenosine A_1 receptors. Inhibition of transmitter release, namely glutamate, selectively depresses excitatory synaptic transmission (Proctor & Dunwiddie, 1992; Thompson *et al.* 1992)

When considering more integrative properties of neuronal circuits, postsynaptic sites of action also have to be accounted. Thus, A_1 postsynaptic receptor activation stimulates potassium conductance leads to neuronal hyperpolarization and controls neuronal firing (Greene & Haas, 1991). Also, A_1 receptors located at the postsynaptic density can influence neuronal responsiveness to excitatory stimuli through a simultaneous control of N-type calcium channels and NMDA receptors (Gomes *et al*. 2010). These findings reinforced the idea that the adenosinergic modulatory role upon NMDA receptor contributions for plasticity and long-lasting plasticity (de Mendonça & Ribeiro, 1997; Klishin *et al*. 1995). In such investigations, it was found that the external application of an adenosine A_1 receptor antagonist increased both the amplitude of excitatory postsynaptic currents and the NMDA receptor-mediated component for the currents (Klishin *et al*. 1995). In isolated rat hippocampal neurons, adenosine A_1 receptor activation inhibits NMDA receptor-mediated currents (de Mendonça *et al.* 1997). Furthermore, this effect occurs at very low concentrations of A_1 agonist, which may imply that there is a tonic inhibitory action of adenosine (Sebastião & Ribeiro, 2009). Accordingly, when an A_1 receptor antagonist is used, NMDA receptor-mediated currents increase, probably due to the recruitment of previous silent NMDA receptors (Klishin *et al*. 1995). Apparently, endogenous adenosine, through a postsynaptic action, is capable of inhibiting voltage and NMDA receptor-sensitive dendritic spikes in CA1 population of neurons (Li &

Henry, 1998). These postsynaptic actions upon NMDA receptor activity together with the ability of A_1 receptors to inhibit glutamate release, are the basis for the adenosine A_1 receptor mediated inhibition of synaptic plasticity phenomena in CA1 and CA3 neuronal populations (Sebastião & Ribeiro, 2009), as it is possible to see in the schematics displayed in figure 2.5.3.2.

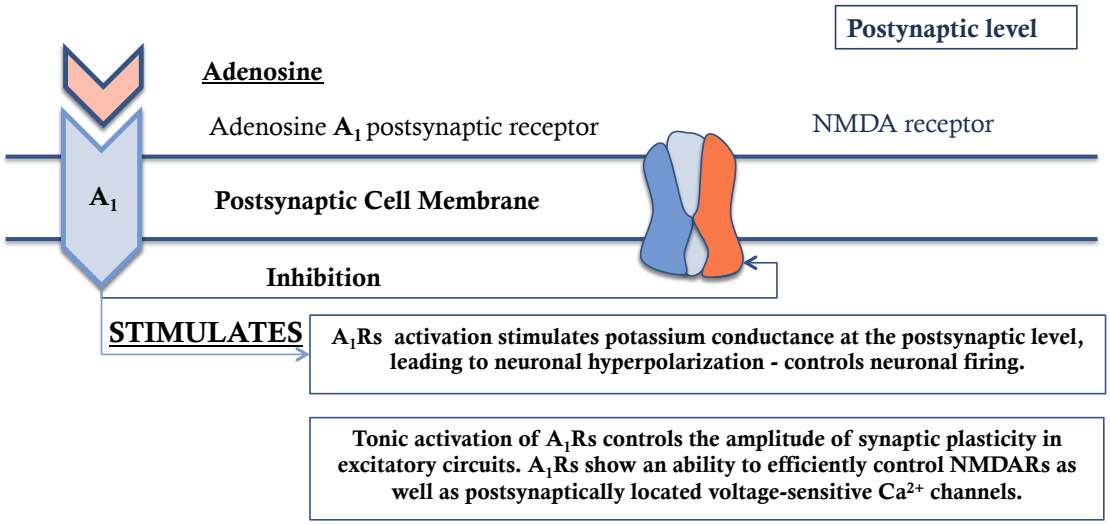

Figure 2.5.3.2 – Postsynaptic modulatory action of adenosine A_1 receptors. A_1 receptors stimulate potassium conductance and effectively control the amplitude of synaptic plasticity in excitatory circuits (Green & Haas, 1991).

During several years the research on A_{2A} adenosine receptor actions in brain function was clouded due to the prominent inhibitory role of adenosine in the control of basal synaptic transmission. The lack of research on A_{2A} receptors was further aggravated by the widespread idea that the distribution of these receptors was limited to specific cerebral areas, namely the striatum (Svenningsson *et al*. 1999). Albeit their distribution in extra-striatal areas appears to be significantly lower than in the striatum, A_{2A} receptors are widely spread in the brain, especially in the synapses (Sebastião & Ribeiro, 1992) and dopamine-enriched areas (Svenningsson *et al*. 1997). After discovering that A_{2A} receptors are expressed in extra-striatal areas, the research of the actions of these receptors upon synaptic transmission increased significantly and new modulatory actions of these receptors were rapidly found. Although these receptors show a limited impact on the control of basal synaptic transmission, they are crucial in controlling synaptic plasticity (Cunha *et al*. 2008). In contrast with their modest role in the control of neurotransmitter release, presynaptic A_{2A} receptors effectively modulate the action of several receptors. This led to the suggestion that

adenosine A_{2A} receptors mostly behave as regulators of other modulatory systems (Lopes *et al.* 1999).

A_{2A} adenosine receptors were found in the rat hippocampus, where it was also observed the binding of specific A_{2A} receptor agonist CGS 21680 (Cunha *et al.* 1996). Accordingly, more recently it was established that CGS 21 680 has excitatory effects on hippocampal pyramidal cells and that are blocked by adenosine A_{2A} receptor antagonist DMPX (Li & Henry, 1998). In addition to data that demonstrated similar results in hippocampal population spikes (Sebastião & Ribeiro, 1992), these results were the first ones suggesting that the excitatory roles of adenosine are mediated via activation of A_{2A} receptors. Also, these data postulated in first hand that the excitatory effects of A_{2A} receptors are expressed via both presynaptic and postsynaptic adenosine receptor activation (Li & Henry, 1998).

At a presynaptic level, A_{2A} receptors are known for playing a facilitatory role upon glutamate release (Lopes *et al.* 1999). Due to a tight A_1-A_{2A} receptor interaction (Lopes *et al.* 1999), it was fairly unclear whether the excitatory role of A_{2A} receptors was to directly control neurotransmitter release or, alternatively, to attenuate the tonic inhibition mediated via adenosine A_1 receptor activation.

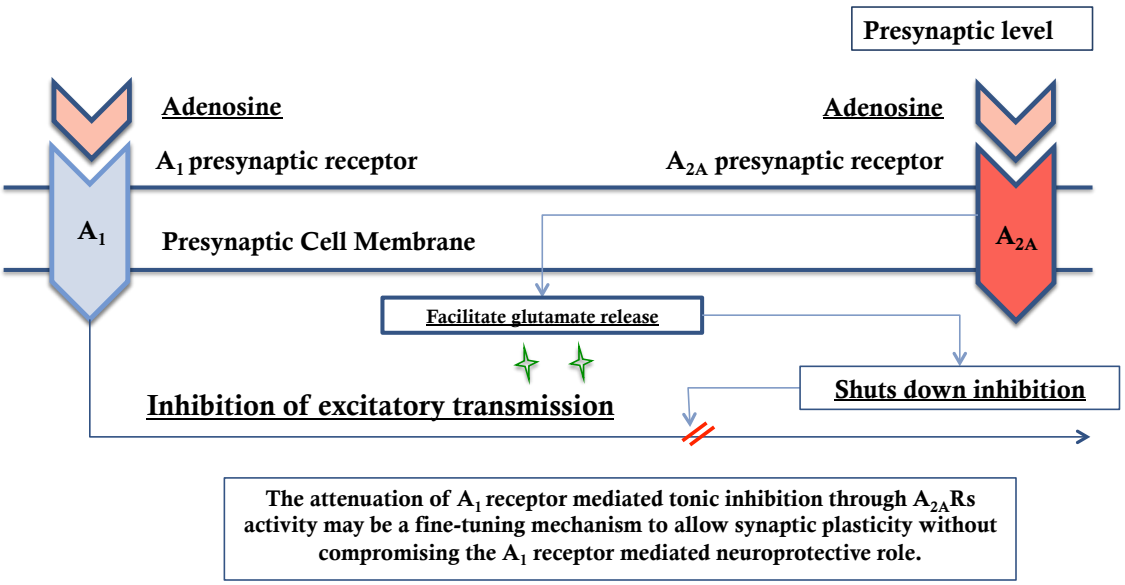

Figure 2.5.3.3 – Presynaptic actions of adenosine A_{2A} receptors. Presynaptic A_{2A} receptors can facilitate glutamate release into the synaptic cleft, only when a tonic A_1 receptor-mediated inhibition is present.

As it is possible to see in figure 2.5.3.3, nowadays it is known that the facilitation of hippocampal synaptic transmission mediated by adenosine A_{2A} receptor

specific agonists it is not a direct effect but rather a consequence of a presynaptic attenuation of A_1 receptor response (Lopes *et al*. 2002). Attenuation of A_1 receptor inhibition seams to constitute a fine-tuning mechanism that allows frequency-dependent phenomena without compromising the A_1 receptor-mediated neuroprotective role (Lopes *et al*. 2002).

At a postsynaptic level, adenosine A_{2A} receptors also display a role in the modulation of excitatory synaptic transmission. The modulation of NMDA receptor activity is of particular interest to this work and it has been a subject recently investigated, especially due to the consequences of NMDA receptor activation for synaptic plasticity processes. It is known that the pharmacological activation of adenosine A_{2A} receptors regulates NMDA receptor function in the striatum (Tebano *et al*. 2005, Wirkner *et al*. 2004). Also, it was recently shown that there are forms of LTP that are mediated by postsynaptic NMDA receptor activity at mossy fibers synapses (Rebola *et al*. 2008). This form of LTP is dependent of elevations in intracellular Ca^{2+} inside the postsynaptic neuron, requiring the co-activation of NMDA receptors and metabotropic glutamate receptor 5 (mGluR5) in addition to adenosine A_{2A} receptors (Rebola *et al*. 2008). Given the role of NMDA receptor activity in synaptic plasticity, memory formation and spatial learning, these results show that postsynaptic adenosine A_{2A} receptors may potentially affect information processing in CA3 neuronal networks and memory performance.

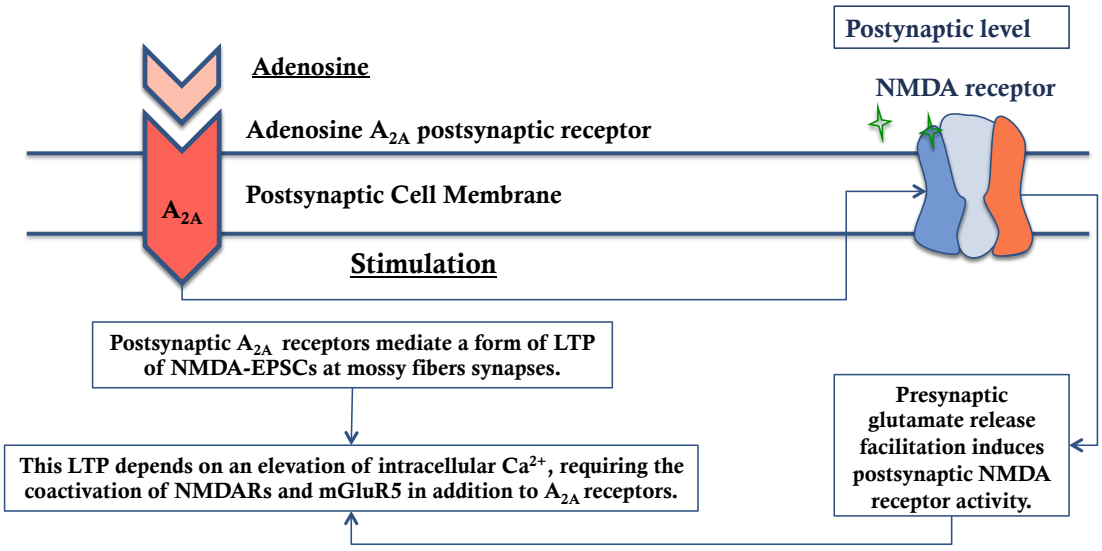

Figure 2.5.3.3 – Postsynaptic actions of adenosine A_{2A} receptors. Modulatory effects of A_{2A} receptors upon NMDA receptor activity at the mossy fibers (Rebola *et al*. 2008).

Whether A_{2A} receptors modulate the activity of NMDA receptor in the hippocampus is still unclear and comprises the main objective of the present work.

Summarizing, at the hippocampus adenosine A_{2A} receptors are located at presynaptic and postsynaptic levels, where they control several key processes for synaptic plasticity and memory formation. Indeed, adenosine A_{2A} receptors are known to directly control the release of glutamate (Lopes *et al*. 2002) as well as the activation of glutamate receptors, such as AMPA receptors (Dias, *et al*. 2012). This work was developed with the main objective of understanding if the activation of excitatory adenosine A_{2A} receptors could also modulate the activity of glutamate NMDA receptors at CA1 pyramidal cells. To achieve these goals, it would be important to create experimental conditions that allow discriminating between a pre or a postsynaptic modulatory interaction between receptors. For that, the patch clamp technique is highly suitable to successfully achieve the objectives proposed for this work.

TECHNIQUES

3

3.1 – Electrophysiological recordings in hippocampal slices

3.1.1 – Tissue preparation

The discovery that brain tissues could be isolated and kept alive and healthy outside the body, triggered physiologic and pharmacologic research in the nervous system. Warburg and colleagues initiated metabolic brain slices studies in 1924 (see Hertz, 2012). In the subsequent decades brain slice preparation was optimized and carried on. In 1966, Yamamoto and McIlwain showed that brain slices could display synaptic activity. Additionally, Richards and McIlwain showed in 1967 that the synaptic activity and excitability of brain tissues kept outside the body in an artificial environment (*ex* vivo) were similar to that observed in *in vivo* tissues.

In hippocampal research, the brain slice preparation has been predominantly used over the years to study synaptic transmission phenomena in various hippocampal neurons and interneurons distributed over different hippocampal populations. The hippocampus lies within the medial temporal human lobe showing a distinctive curved shape. Its general form is kept among various mammalian species, although its

morphology does vary. Hippocampal formation is a bilateral limbic structure that in the rat brain has a semi-circular form. Its intact extraction from the brain is very easily achieved. Usually, after extraction, both hippocampi are transversely sectioned.

The internal structure of the hippocampus can be macroscopically described as two C-shaped cell layers that are interlocked: the granular cell layer of the dentate gyrus and the pyramidal cell layer of the Ammon's horn (hippocampus proper) and the subiculum (Lopes da Silva *et al*. 1990). The hippocampus proper is composed of various subfields. The *cornus ammonis* (CA1) is generally differentiated into CA1, CA2 and CA3 areas and it showing seven clearly defined depth layers: *stratum moleculare*; *stratum lacunosum*; *stratum radiatum*; *stratum pyramidale* (the principal cell layer); *stratum oriens*; *alveus a*nd *epithelium*. The dentate gyrus is composed by three layers: *stratum moleculare*; *stratum granulosum* (principal cell layer) *and stratum polymorphum*.

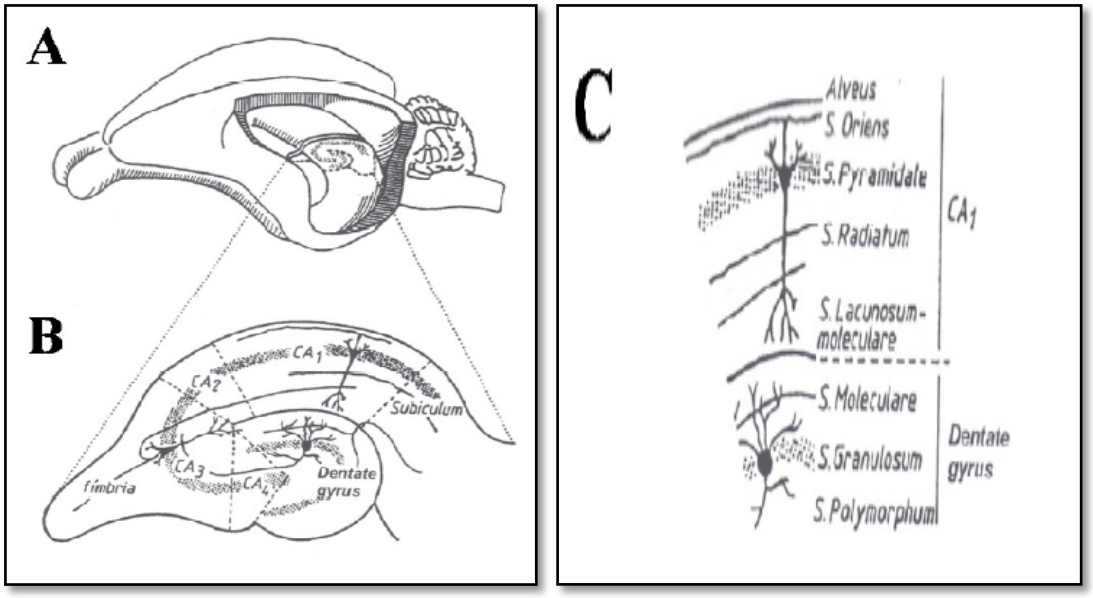

Figure 3.1.1.1 – Hippocampal anatomy. A) Localization of hippocampus in the rat brain. B) Schematic representation of a transversal hippocampal slice with indication of the different areas and the main excitatory circuit. C) Representation of a section of CA1 and Dentate Gyrus (DG) area with the indication of the different layers. Adapted from Andersen et al., 1971 and Amaral and Witter, 1989. Obtained from Rombo, 2009.

Ramon y Cajal first hippocampal drawings showed the major pathways of signalling flow through the hippocampal formation. Later, Per Andersen described the trisynaptic circuit or trisynaptic loop (Andersen *et al*. 1971). This loop comprises the

hippocampal circuitry that connects the dentate gyrus, CA1 and CA3 areas. From the dentate gyrus granule cells, efferent fibers project and send information onto CA3 pyramidal cells. In turn, these cells send their axons to CA1 pyramidal cells through a set of fibers acknowledged as the Schaffer collaterals. From CA1 area, axons project mainly to the entorhinal cortex (Dias, 2011).

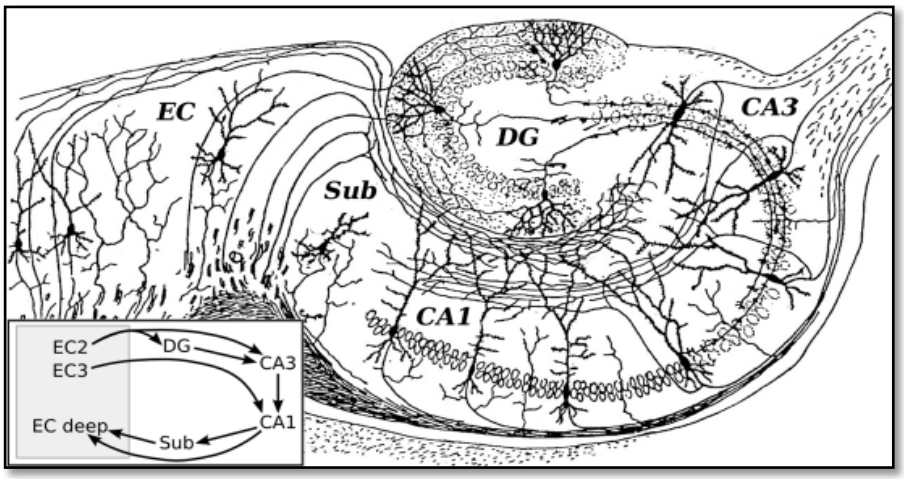

Figure 3.1.1.2 – Hippocampal structure. The two interlocked "C" shaped curves are well visible. The drawing contemplates also the cell types through which information flows to form the trisynaptic circuit. DG: dentate gyrus; Sub: Subiculum; EC: entorhinal cortex. This drawing is modified from Ramon y Cajal, 1909. Obtained from Dias, 2011.

Per Andersen also found that all the important hippocampal connections were maintained when the hippocampus was cut transversally to its long axis (Andersen *et al*. 1969). This was later the basis for the introduction of hippocampal slice preparations by Skrede and Westgaard (1971). Hippocampal slice preparations preserve the main physiological circuits intact, 400 to 500 μm thick transverse slices can keep the tissues alive and healthy for several hours, when properly oxygenated in a bath of artificial cerebrospinal fluid. The thinness of the hippocampal slices allows the access to the try-synaptic circuit. The hippocampal circuitry and neuronal laminar organization is fully preserved and maintained in slices preparation. Hippocampus slice preparation has several advantages for the investigator. In the study of central nervous system activity, hippocampal slices allow the investigator to directly control the surrounding environment of the tissue as well as to apply drugs to the all slice or just to specific regions, thus allowing the investigator to directly control and manipulate pharmacological tools in known concentrations. These drugs can be subsequently washed out or withdrawn at anytime. Also, for electrophysiological

procedures, recording and stimulation electrodes can be accurately placed upon specific regions of interest. Together with the development of cutting solutions which composition minimizes the excitotoxic insult during slice preparation, the introduction of vibrating microtomes for tissue processing greatly increased the success of patch-clamp recordings from brain slices (see Aitken *et al*. 1995).

3.1.2 – Patch-clamp techniques

It was in the late seventies that Neher and Sakmann (1976) monitored in first hand the opening and closing of single ion channels located in cell membranes through conductance measurements. These first recordings of single-cell currents were obtained from denervated frog muscle fibers, using glass pipettes of narrow tip (Neher & Sakmann, 1976). The investigators noted that when the tip of the pipette touched the surface of the cell membrane it developed high resistances, in order of 50 MΩ. The high resistance between tip and surface was called "seal" and consequently patch-clamp was initiated. Briefly after the establishment of this technique, Sigworth and Neher (1980) discovered that the appliance of gentle suction to the tip of the pipette could create a tight and stable mechanic contact whit a resistance of 10-100 GΩ (hence, giga-seal). This giga-seal considerably reduced the background noise allowing also grater time resolution.

The high resistance of the seal ensures that virtually all the current from the membrane flows into the pipette interior. To obtain a giga-seal became the first and mandatory step of patch-clamp recordings. Initially, recordings were obtained from the small membrane portion that was pulled inside the pipette after suction appliance. This way, it was possible to measure acutely the current flow through the trapped ion-channels. This patch-clamp configuration received the name of "cell-attached recording". Although there is still a lot of work that it is done in cell-attached mode, this configuration cannot provide any information of the resting potential of the cell, nor are the extra- or intra-cellular solution changed easily (see Dias, 2011). Fortunately it was found that the membrane portion contained inside the pipettes tip could be gently removed through the application of more suction. Once this happens, the electrode can achieve direct electrical contact with the entire cell interior. Consequently, the voltage across all the cell membrane could be clamped, instead of just the little portion of the patch. The whole-cell and perforated patch modes allow

the measurement of an entire ion-channel population located in the cell membrane, therefore giving current flow readings of all cellular activity mediated by the specific ion-channels (for review see Liem *et al*. 1995). This patch-clamp configuration came to be known as "whole-cell recording" and is by far the most widely used patch-clamp configuration. However, it is curious that, by definition, the whole-cell configuration does not fit into the strict patch-clamp definition, as in it the clamp is applied to the entire cell and not to a small patch of the membrane (Dias, 2011).

Voltage-clamp procedures are often used to study current flows through specific ion-channels. In order to achieve these goals it is very important to effectively block undesired currents mediated by ion-channels out of the range of the study interest. This can be attained by selectively choosing the ionic composition of the bath solution, by controlling the holding potential of the cell membrane or through the use of pharmacological tools that specifically block or induce the activity of certain individual or group ion-channels (for review see Sontheimer & Olsen, 2007). This was the procedure used in this work, where ionic current through NMDA receptor ion channels, have been record in the whole cell patch-clamp configuration.

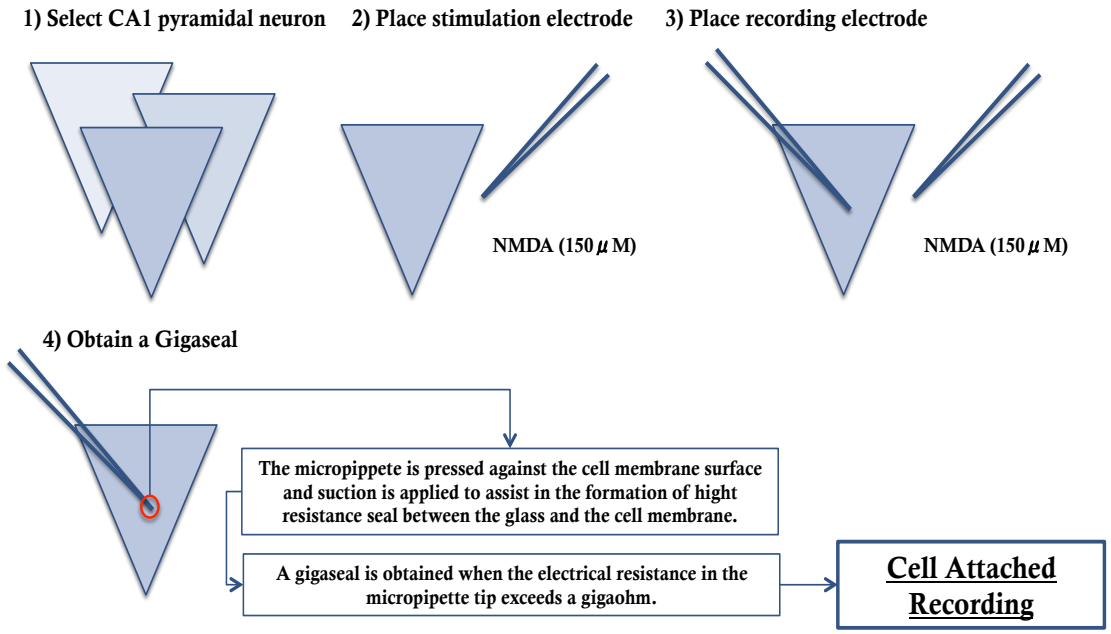

Figure 3.1.2.1 – Step by step to achieve NMDA receptor-evoked postsynaptic currents recordings. Steps 1 to 4: Positioning of the stimulation and recording electrodes and obtaining cell-attached recording; Step 5 – Patch burst through appliance of more negative pressure obtaini8ng whole-cell recording configuration; Step 6 – Stimulation with 2 minute intervaled NMDA puffs and recording of the NMDA receptor-evoked postsynaptic currents.

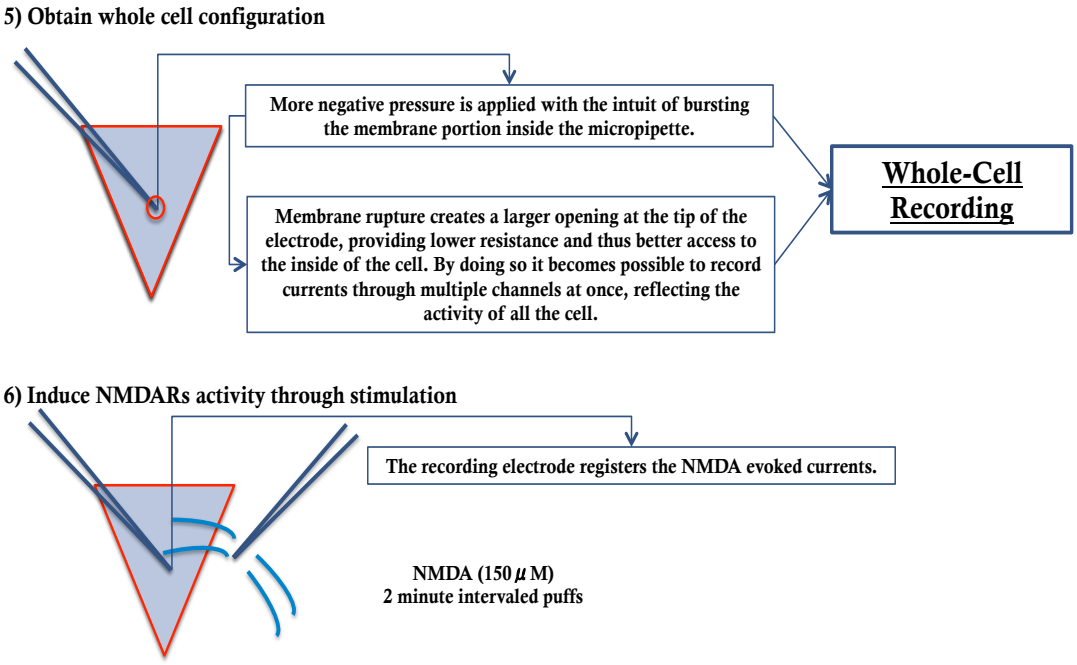

Figure 3.1.2.2 – Step by step to achieve NMDA receptor-evoked postsynaptic currents recordings. Step 5 – Patch burst through appliance of more negative pressure obtaini8ng whole-cell recording configuration; Step 6 – Stimulation with 2 minute intervaled NMDA puffs and recording of the NMDA receptor-evoked postsynaptic currents.

METHODS

4

4.1 – Animals

All the experiments were conducted with adult male Wistar rats aged between 5 and 10 weeks (Harlan Iberica, Spain). The animals were kept under standardized temperature, humidity and lighting conditions, according to Portuguese Legislation. Animals were housed in the local Animal House in 12 hours light/dark cycle and were provide with food and water *ad libitum*. For ethical reasons, care was taken in order to assure that the number of rats utilized during this investigation was the smaller possible.

4.2 – Preparation of hippocampal slices for electrophysiological recordings

Before dissection the animals were anesthetized with Isoflurane (in 1,2-Propylenglycol 50% (v/v)) in a proper anesthesia chamber. To assure that the animal is properly anesthetized the animal behavioural responses were observed. The first indications of anesthesia include the lack of movement and the slow respiratory rate. The skin was then pinched and the absence of movement reflected deep anesthesia. However, it is very important that the animal does not die under anesthesia, since it would exacerbate neuronal cell death processes, by decreasing the time window between sacrifice, brain extraction and its placement in a artificial cerebrospinal fluid solution. After proper anesthesia the animal was sacrificed by decapitation in a guillotine. Subsequently, the brain was rapidly extracted from the skull and placed in an oxygenated iced artificial cerebrospinal solution (aCSF solution) with the following composition (in mM): sucrose 110; KCl 2.5; $CaCl_2$ 0.5; $MgCl_2$ 7; $NaHCO_3$ 25 NaH_2PO_4 1.25; glucose 7, oxygenated with 95% O_2 and 5% CO_2, pH 7.4. Together with use of this solution, a quick dissection under low temperature reduces the probability of neuronal cell death, increasing the viability of the tissue and cells. After the brain was extracted and hemisected, both hippocampi were dissected and used to obtain 300μm thick transversal slices cut on a vibrating microtome acknowledge as Vibratome (VT1000 S; Leica; Germany) in the same ice cold dissecting aCSF solution.

After both hippocampi were cut on the Vibratome the slices were incubated for 30 minutes in a 35ºC aCSF bath solution containing (in mM): NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2 and glucose 10, pH 7.4, gassed with 95% O_2 and 5% CO_2. After the 30minute window time, the slices were withdrawn from the 35ºC bath and placed in recovering at room temperature for at least one hour.

Another solution was prepared for use during the patch-clamp procedures with the following composition (in mM): NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; Glycine 5; $CaCl_2$ 2 and glucose 10, pH 7.4. The composition of this solution was very similar to the recovery one, except that it did not included $MgSO_4$. The omission of Mg^{2+} was required NMDA receptors are blockade by extracellular magnesium, whenever the membrane potential is close to the resting state. Indeed, in experiments conducted using the standard aCSF solution, the NMDA receptor evoked postsynaptic potentials were almost inexistent at membrane potentials of -60 mV. Upon the

withdrawn of Mg^{2+} and the inclusion of glycine (NMDA receptor co-agonist) it was possible to record NMDA receptor evoked postsynaptic potentials.

After the recovery period the slices were ready to be used in patch-clamp techniques. For that, individual slices were fixed with a grid in a recording chamber (5-6mL) and continuously superfused by a gravitational superfusion system at 2-3mL/min with aCSF (with glycine and without Mg^{2+}) at room temperature. The pharmacological tools added during the procedure reached the slice within 2 or 3 minutes after its application.

Whole-cell recordings were obtained from CA1 pyramidal cells. CA1 pyramidal cells were visually identified using a Carl Zeiss Axioskop 2FS upright microscope (Jena, Germany) equipped with infrared video microscope and differential interference contrast optics. Just to ensure that the cells recorded were in fact CA1 pyramidal cells at the beginning of some experiences a fire-pattern was routinely performed. Pyramidal cells are known to express a slow fire frequency (≤5Hz), longer action potentials (≥0,8ms) and featuring spike-frequency adaptation.

Patch pipettes were made from borosilicate glass capillaries (1.5mm outer diameter, 0.86 inner diameter, Harvard Apparatus) on a pipette puller (PC-10 Puller, Narishige Group). The resistances of the recording electrodes/pipettes varied between 4 and 9MΩ, with its optimal resistance located in 6-7MΩ. To patch the cell the pipettes were filled with an intracellular solution containing in its composition the following (in mM): K-gluconate 125; KCl 11; CaCl2 0.1; MgCl2 2; EGTA 1; HEPES 10; MgATP 2; NaGTP 0.3 and phosphocreatine 10, pH 7.3, adjusted with KOH (1M), 280-290mOsm.

Whole-cell NMDA-evoked postsynaptic currents (PSCs) were recorded in a voltage-clamp mode (Vh =-60mV) with an EPC-7 amplifier (List Biologic, Campbell, CA). Offset potentials were nulled directly before giga-seal formation. The holding current was monitored throughout the experiment and when this parameter varied by more than 20%, the experiment was rejected. Voltage errors caused by small changes in input resistance were not corrected. The current signal was filtered using a 3 and 10 kHz three-pole Bessel filter of an EPC-7 (List Medical) amplifier.

NMDA receptor-mediated currents were evoked using a pressure ejection system (PicoPump PV820; World Precision Instruments). A micropipette containing NMDA (150μM) was positioned near the recording cell and pulses of pressure were applied (10ms puffs, 6-10psi, applied at 2min intervals). The experimental protocol

was initiated at least 30min after the whole-cell formation in order to assure the stabilization of the elicited currents. Application of a selective NMDA receptor agonist (NMDA 150µM) through 2min intervaled puff stimulation onto the soma of CA1 pyramidal cells was used to evoke currents that reflected the activity of NMDA receptors. This kind of protocol has the advantage of allowing the subsequent application of pharmacological tools of interest. In this case, selective adenosine A_{2A} receptor agonist and antagonist (CGS 21680 and SCH 58261, respectively), specific NMDA receptor antagonist (DL-APV) and the competitive antagonist of other glutamate receptors, such as kainate and AMPA were applied. The continuous measurement of NMDA receptor-evoked postsynaptic currents throughout the experiment guarantees that any effect observed upon the registered currents is a consequence of the drug being applied. Panels A, Band C displayed in figure 4.2.1 show the placement of the recording and stimulation electrodes first in CA1 hippocampal region and then patching a pyramidal neuron. Panel D displays an example of a NMDA-evoked PSCs recorded during the procedure.

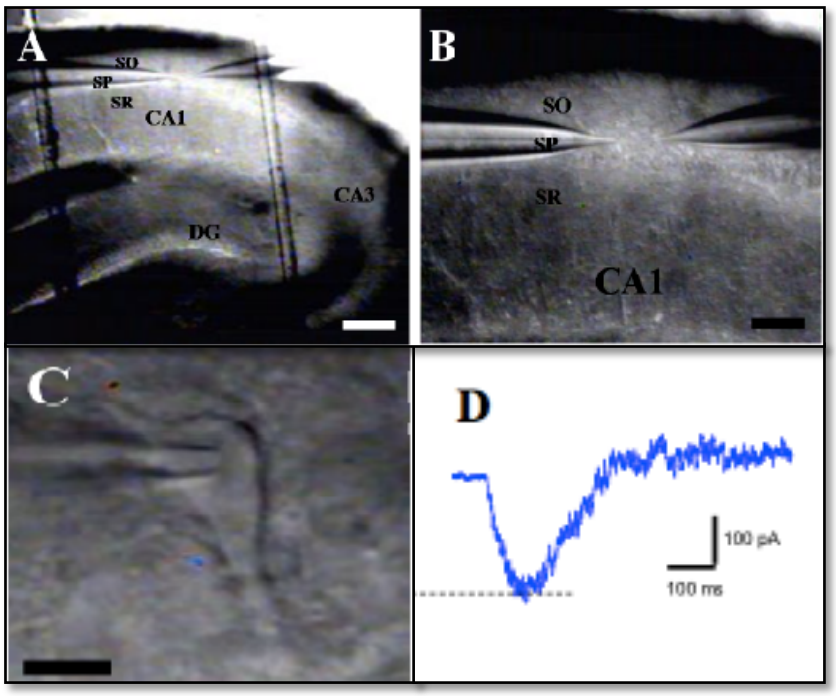

Figure 4.2.1 – Patch-clamp recordings of NMDA receptor-evoked postsynaptic currents (PSCs). A) The recordings were effectuated in *stratum pyramidale* (SP) of CA1 pyramidal cells in rat hippocampal slices. B) This picture shows the disposition of the recording (left) and the stimulation (right) electrodes. Note that the recording electrode is deeper into the slice than the stimulation one. C) Patching a CA1 pyramidal neuron. D) NMDA receptor-evoked postsynaptic currents being elicited by intervaled puff stimulation of NMDA (150µM). Calibration: A – 300µm; B – 120µm; C – 10µm D – 400ms, 100pA. Photos A, B, C were adapted from Rombo, 2009.

Data acquisition was under the control of pCLAMP 10 Electrophysiology Data Acquisition and Analysis Software program (Axon Instruments Cellular Neurosciences product line of Molecular Devices Corp.). Data analysis was executed with Clampfit 10 (included in pCLAMP 10). Graph processing and statistic analysis were performed in Prism Version 6.00 for Mac OS X (GraphPad Software).

4.3 – Pharmacological tools

NMDA (N-methyl-D-aspartic acid) was obtained from Ascent Scientific Ltd (Bristol, UK); CGS 21680 (2-[4-(2-p-carboxyethyl)phenylamino]-5'N-ethylcarbozamidoadenosine) that is a selective A_{2A} receptor agonist and SCH 58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5 c]pyrimidine) that works as a selective A_{2A} receptor antagonist, were obtained from Sigma-Aldrich (MO,USA). TTX (tetrodoxina citrate; Sodium-channel blocker), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), that acts as an antagonist of competitive AMPA/kainate glutamate receptors, was obtained from Tocris Bioscience (Bristol, UK) and DL-APV (2-amino-5-phosphonovaleric acid), that is a selective NMDA receptor antagonist, was obtained from Ascent Scientific Ltd (Bristol, UK). Isoflurane was obtained from Abbot Laboraties (Barcelona, Spain) and 1,2-Propylenglycol from Merck (NJ, USA). CGS 21680 (5mM) and CNQX (100 mM) were prepared as a stock solution in dimethylsulfoxide (DMSO). TTX (1mM) was prepared in water as well as NMDA (100 μM) and DL-APV (50 mM). Stock solutions were aliquoted and stored at -20°C until use. Dilutions of these stock solutions to the final concentration were made freshly before each experiment.

4.4 – Data analysis

Values refer to mean peak amplitude of NMDA-evoked macroscopic currents and are represented as mean ± Standard Error of the Mean (SEM) from n experiments. Statistical significance was assessed by a two-tailed Student's t test for the experimental versus control condition. A p value of 0.05 or less was considered to account for significant differences. Statistical analyses were conducted with the Prism Version 6.00 for Mac OS X (GraphPad Software).

RESULTS
5

Although there is a great deal of research involving A_{2A} actions on hippocampal synaptic transmission, the modulatory roles of A_{2A} receptors are still elusive. In hippocampal CA1 pyramidal neurons there are evidences that A_{2A} receptors modulate the activity of AMPA receptors (Dias *et al*. 2012), but it is unclear if these receptors are able to modulate the activity of NMDA receptors in this hippocampal population.

5.1 - Activation of adenosine A_{2A} receptors facilitates NMDA receptor-evoked postsynaptic currents in CA1 pyramidal neurons

In the first set of experiments the initial step was to achieve a steady and stable baseline of NMDA-evoked PSCs amplitudes. For that, NMDARs-mediated currents were evoked through pressure application (*puff* stimulation) of NMDA (150μM) (specific NMDAR agonist) directly onto the cell soma of the selected pyramidal neuron. The interval between each pressure application of NMDA was of two minutes and the baseline was always obtained after, at least, 5 consecutive *puffs* (10 minutes). When a stable baseline of NMDA-evoked PSCs was successfully obtained, the selective A_{2A} receptor agonist CGS 21680 (30nM) was applied to the bath solution during 25 *puffs* (50 minutes). The CGS 21680 stock solution was prepared at a concentration known to be selective for adenosine A_{2A} receptors, namely 30nM (Jarvis *et al*., 1989). It is also known that at this specific concentration CGS 21680 activates A_{2A} receptors in hippocampal slices (Sebastião & Ribeiro, 1992). Additionally, it is known that CGS 21680 has excitatory effects on hippocampal pyramidal cells (Li & Henry, 1998) and that it enhances AMPA-evoked currents in CA1 pyramidal neurons (Dias *et al*. 2012). As shown in figure 5.1.1, CGS 21680 (30nM) enhanced NMDA-evoked currents in CA1 pyramidal cells. As can also be concluded from the time course of CGS 21680 (figure 5.1.2), the facilitatory effect started to be observed at about 5-10 minutes and the full effect occurred about 40 minutes after CGS 21680 addition. Even during the perfusion of CGS 21680, it was evident that the PSCs started to decrease probably due to A_{2A} receptor

desensibilization. Thus, at 30-40 min after GCS 21680 application (dark pink area in figure 5.1.2), the NMDA-evoked PSCs amplitudes were 135&±8.8% of baseline, whereas in the last ten minutes before washout, the PSC amplitude was 123±4.7% of baseline. Both values are statistically different from the control (before CGS 21680).

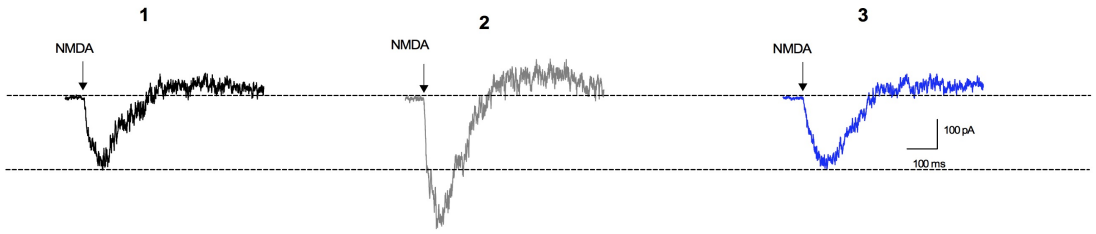

Figure 5.1.1 – Illustrative current recordings of a representative experiment. The numbers in the currents represent the phases of the experiments identified in **figure 5.1.2, panel A**. The arrows identify the moment of brief (10 ms) applications of NMDA (150µM) into the cell soma. 1) PSC evoked 10 minutes before the application of CGS 21680; 2) PSC evoked during the last 10 minutes of CGS 21680 superfusion; 3) PSC evoked 20 minutes after the withdrawn of CGS 21 680 – washout phase. The NMDA-evoked PSC return to values close to the ones measured in the baseline phase.

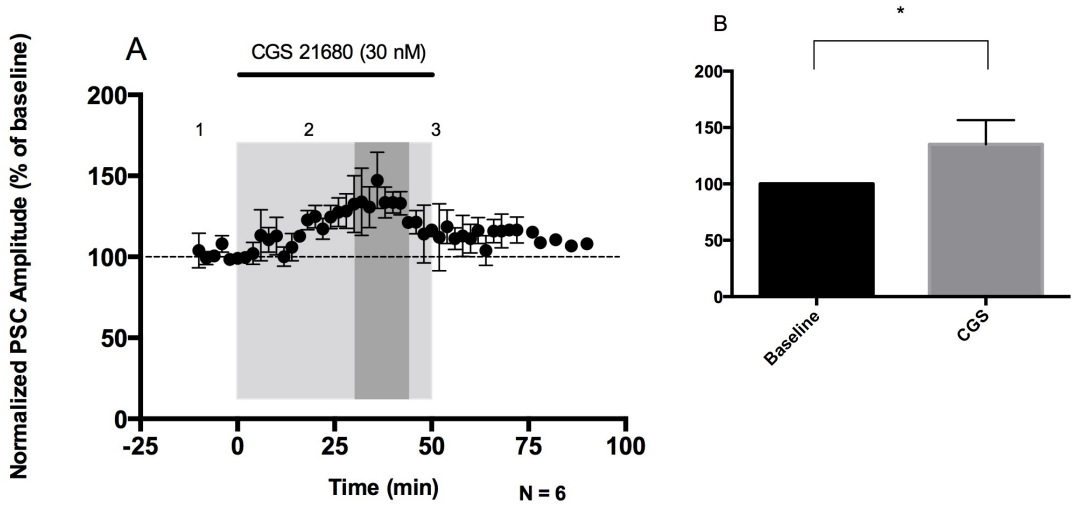

Figure 5.1.2 – Adenosine A_{2A} receptor agonist CGS 21680 modulates NMDA evoked-PSCs in CA1 pyramidal neurons. A) Time course plot of measured currents obtained from 6 experiments displaying that, during the time indicated by the horizontal bar (50 minutes illustrate in the area highlighted in light grey), selective A_{2A} receptor agonist CGS 21680 (30nM) affects NMDA evoked-PSCs recorded from CA1 pyramidal cells (Vh=-60mV). NMDA-evoked PSCs were evoked and measure with two-minute intervals. For statistical analyses, the PSCs were normalized to the 10 minutes before CGS 21680 (30nM) application (100% = 480,1±63,9 pA). Significant statistical differences were found between current amplitude before and 50 minutes after CGS 21680 (30nM) application: 123%±4,7% of baseline, n=6, p<0.005, paired Student's t test. **B)** The full effect occurred about 40 minutes after the application of CGS 21680 (dark grey area in panel **A**). In this period the NMDA-evoked PSCs increased to 135%±8.8% of baseline n=6, p<0.05.

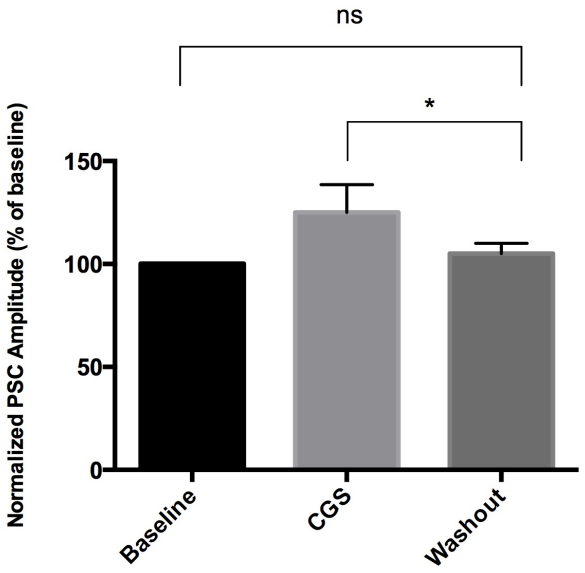

Figure 5.1.3 – CGS 21860 withdrawn significantly altered NMDA-evoked mean peak PSCs amplitudes. B) PSCs mean peak amplitude during the washout procedure decreased significantly, when compared with the last 10 minutes of CGS 21 680 application. Upon CGS 21680 washout, there was a 20,0%±12,0% decrease in average NMDA-evoked PSCs amplitude to 105%±2.5% of baseline values. There are no significant statistical differences when comparing the mean differences between the baseline period and the washout period: 105%±2.5% of baseline, n=4 p>0,05 (100% = 483,3 ± 93,3 pA).

The effect of CGS 21680 (30nM) was fully reversible upon washout. As it is shown in figures 5.1.3, in the experiments in which it was possible to withdrawn the agonist from the solution in a washout procedure, NMDA-evoked PSCs peak amplitudes decreased significantly, when compared with the last 10 minutes of CGS 21 680 application. In the time course plot displayed in figure 5.1.2, it can be observed that NMDA-evoked PSCs in the last 10 minutes of the washout procedure decreased to values really close to the baseline: 105%±2.5% of baseline, n=4 p>0,05. Upon CGS 21680 washout, there was a 20%±12% decrease in average NMDA-evoked PSCs amplitude.

The results of this first set of experiments strongly suggest that adenosine A_{2A} receptors are responsible for a modulation of NMDA-evoked PSCs in CA1 pyramidal cells, this modulation being a potentiation of NMDA-evoked PSCs.

5.2 – Endogenous A_{2A} receptors does not contribute for the measured NMDA-evoked PSCs

To better characterize and describe the effect of the A_{2A} agonist upon NMDA-evoked PSCs, it was considered important to understand if the endogenous adenosine present in the cell environment contributes for the increase in the PSCs. The use of the selective A_{2A} antagonist SCH 58261 (100nM) blocks the possible action of endogenous adenosine and allows comparing a period of baseline without the presence of any drug with a period where the antagonist is present.

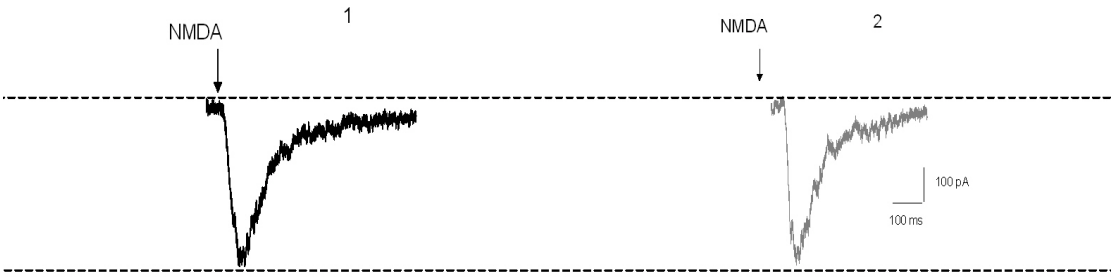

Figure 5.2.1 – Illustrative current recordings. The numbers in the currents represent the two different periods of the experiments identified in the time course displayed in **figure 5.2.2**. The arrows are signalling the moment of brief (10 ms) applications of NMDA (150μM) into the cell soma. 1) PSC evoked 10 minutes before the application of SCH 58261 (100nM); 2) PSC evoked during the last 10 minutes of SCH 58261 (100nM) superfusion.

Figures 5.2.1 and 5.2.2 show that there were no significant differences between the NMDA-evoked PSCs measured before and after the superfusion of A_{2A} receptor antagonist SCH 58261 (100nM). The statistic analysis showed that there were no statistically significant differences between both periods, with average NMDA-evoked amplitude currents being 103%±14,5% of baseline, n=3, $p>0.05$. These data suggest that, with the recording conditions used in these experiments, endogenous adenosine does not regulate NMDA receptor responses

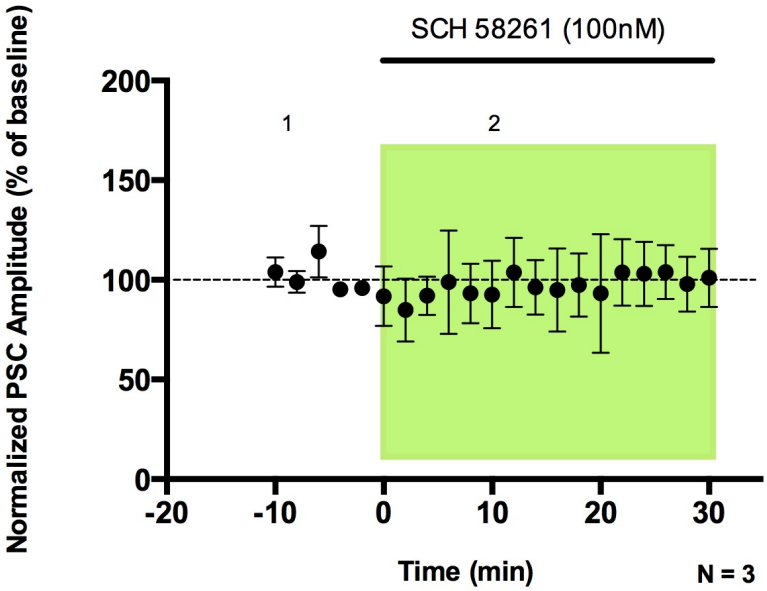

Figure 5.2.2 - Endogenous A_{2A} receptor activity does not contribute for the measured NMDA-evoked PSCs. Time course plot of measured currents obtained from 3 experiments displaying that, during the time indicated by the horizontal bar (30 minutes, green area), the selective A_{2A} receptor antagonist SCH 58261 (100nM) does not affect NMDA evoked-PSCs recorded from CA1 pyramidal cells (Vh=-60mV). NMDA-evoked PSCs were evoked with two-minute intervals. For statistical analyses, the PSCs were normalized to the 10 minutes before SCH 58261 (100nM) application (100% = 243,6±47.0 pA). No significant statistical differences were found between current amplitude peaks before and 30 minutes after SCH 58261 (100nM) application: 102,8%±14,5% of baseline, n=3, $p>0.05$, under Student's *t* test.

5.3 - Previous application of adenosine A_{2A} receptors antagonist blocks the effect of adenosine A_{2A} receptors agonist

A classic pharmacological control was designed to assure that the increase registered in NMDA-evoked PSCs after the administration of CGS 21680 was due to A_{2A} receptor activity. For that, the selective adenosine A_{2A} receptor antagonist SCH 58261 (100nM) was superfused in the bath solution before (at least 10 minutes) the addition of A_{2A} receptor agonist CGS 21680 (30nM). The reason behind this protocol was that any effect mediated by A_{2A} receptors would be prevented by the presence of

the antagonist since previous application of the antagonist at superior concentrations would occupy the A_{2A} receptors, preventing the binding of the A_{2A} receptor agonist.

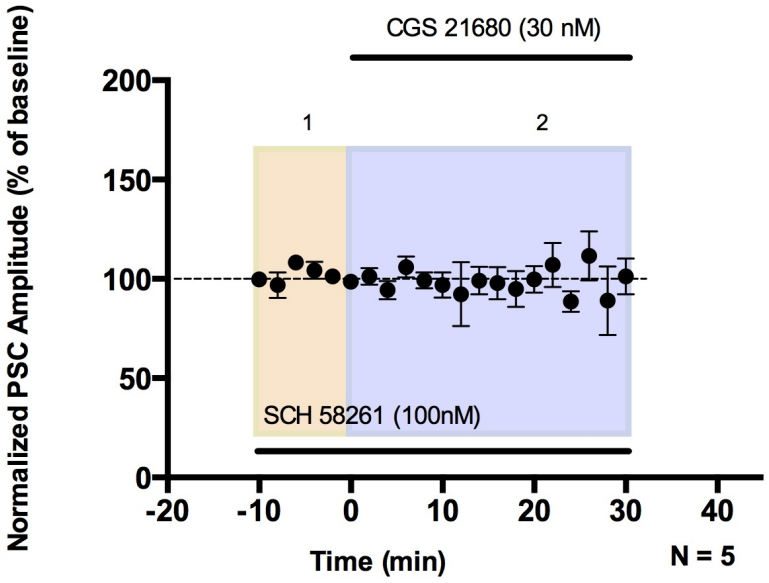

Figure 5.3.1 - Previous application of adenosine A_{2A} receptors antagonist blocks the effect of adenosine A_{2A} receptors agonist. Time course plot of measured currents obtained from 5 experiments displaying that, during the time indicated by the horizontal bar located at the top of the graph (30 minutes, blue area) and in the presence of selective A_{2A} receptor antagonist SCH 58261(100nM), the selective A_{2A} receptor agonist CGS 21680 (30nM) does not affect NMDA evoked-PSCs recorded from CA1 pyramidal cells (Vh=-60mV). NMDA-evoked PSCs were evoked with two-minute intervals. For statistical analyses, the PSCs were normalized to the 10 minutes before CGS 21680 (30nM) application (100% = 336.9±133.0 pA). No statistical significant differences were found between current amplitude peaks before and 30 minutes after CGS 21680 (30nM) application: 102,2%±9,0% of baseline, n=5, p>0.05, under Student's t test.

As illustrated in the time course displayed in figure 5.3.1 and in data represented in figure 5.3.2, in the presence of the selective A_{2A} receptor antagonist the A_{2A} receptor agonist was clearly devoid of effect upon NMDA-evoked PSCs (102,2%±9,0% of baseline, n=5, p>0.05).

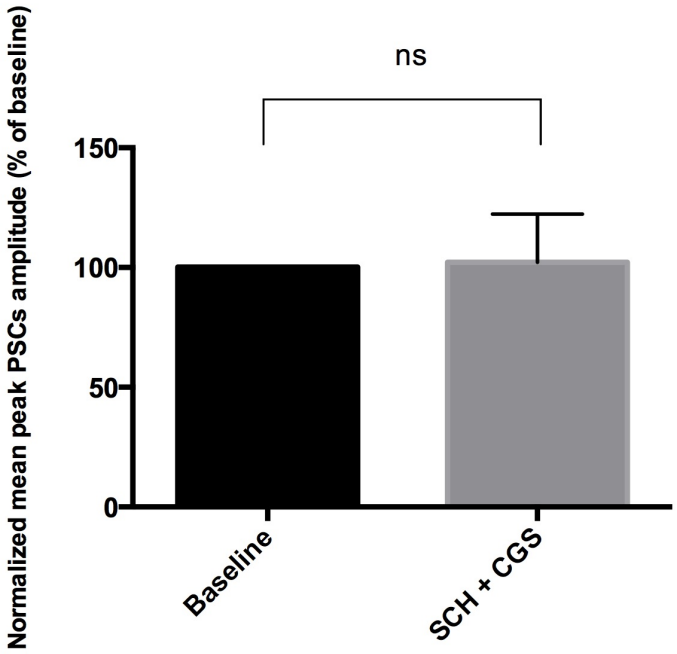

Figure 5.3.2 – SCH 58261 and CGS 21680 simultaneous application did not altered the mean peak amplitude of NMDA-evoked PSCS. Mean effect of selective A_{2A} receptor agonist CGS 21680 (30nM) superfusion in the presence of previously administrated selective A_{2A} receptor antagonist SCH 58261 (100nM) on peak amplitude of NMDA-evoked PSCs from CA1 pyramidal cells. PSCs peak amplitude during the last 10 minutes of the CGS 21680 (30nM) application in the presence of SCH 58261 do not significantly differ when compared with the last 10 minutes of SCH 58261 (100nM) individual application: 102,2%±9,0% of baseline, n=5, p>0.05, paired Student's t test.

5.4 - Application of AMPA/kainate antagonist does not alter the evoked PSCs

Until now the pharmacological controls made to further describe and characterize the nature of the A_{2A} receptor agonist CGS 21680 modulatory effect upon postsynaptic currents evoked by NMDA receptor activity were focused only in the adenosinergic component of the effect. We next addressed if the NMDA-evoked PSCs being measured mainly reflect NMDA receptor activity. Therefore, specific drugs to block the activity of other ionotropic glutamate receptors (AMPA and kainate receptors) were used. A selective competitive antagonist for AMPA and kainate receptors, CNQX (10μM) was chosen to selectively block the activity of these receptors.

As it is possible to observe in figure 5.4.1 the administration of a competitive AMPA/kainate antagonist did not significantly alter the mean peak amplitudes of

NMDA-evoked PSCs (99.0%±2.9% of baseline, n=3, p>0.05). These data suggest that these glutamate receptors did not significantly contribute for the postsynaptic currents that were being measured.

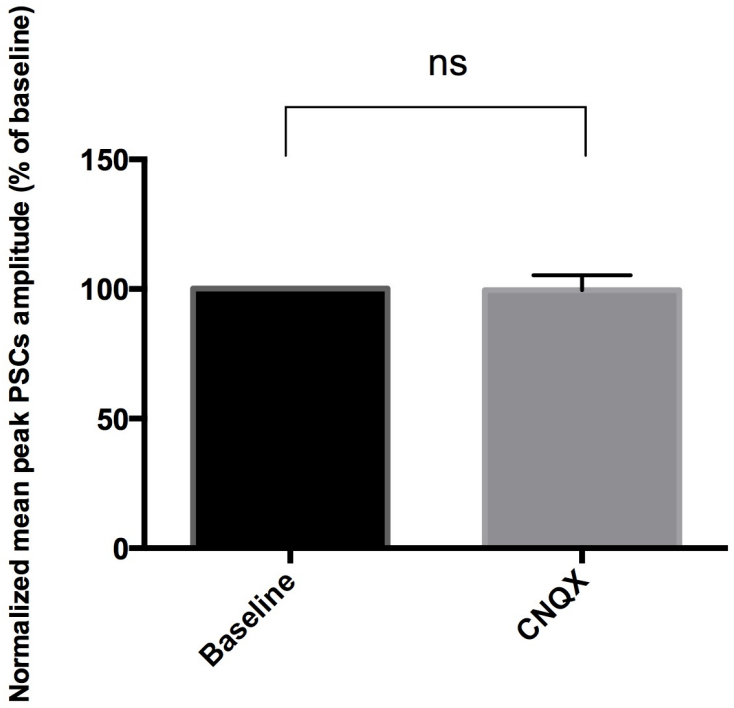

Figure 5.4.1 – AMPA/kainate competitive antagonist CNQX did not altered the mean peak amplitude of NMDA-evoked PSCs. Effect of AMPA/kainate receptor antagonist CNQX (10μM) on mean peak amplitude of NMDA-evoked PSCs from CA1 pyramidal cells. Mean peak PSCs amplitudes during the last 10 minutes of CNQX (10μM) application do not significantly differ, comparing with the baseline: 99.5%±3.3% of baseline, n=3, ns. p>0.05, paired Student's t test (100% = 132,7±11,6 pA).

5.5 - Application of selective NMDA receptor antagonist cancels the evoked PSCs

After observing that AMPA and kainate glutamate receptors are not accountable for the currents that were measured, one more step was taken to make sure that only NMDA receptors contributed for the elicited postsynaptic currents. DL-APV (50μM) is a selective NMDA antagonist that competitively inhibits glutamate binding to the allosteric site of NMDA receptors. A substantial decrease, if not abolishment, of the PSCs being measured was expected if in fact these currents were being mediated by NMDA receptors.

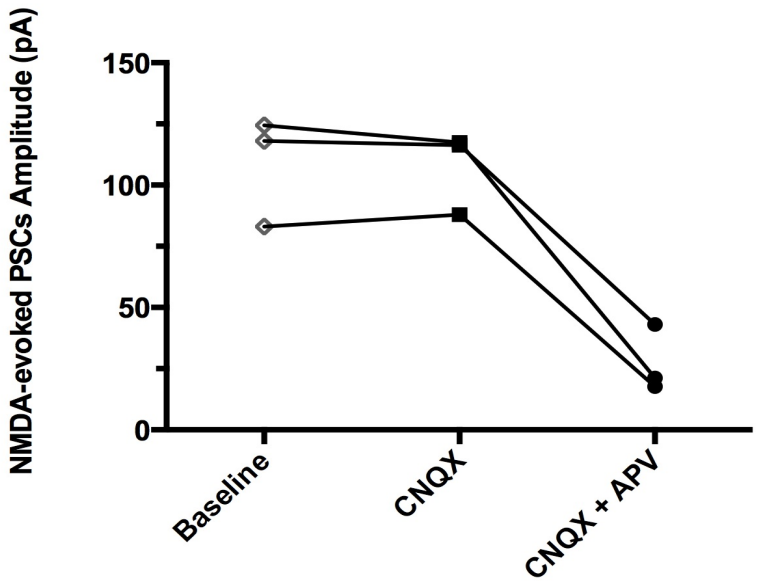

Figure 5.5.1 – NMDA receptor selective antagonist significantly reduces peak amplitudes of NMDA evoked-PSCs. Effect of NMDA receptor antagonist DL-APV (50μM) on peak amplitude of NMDA-evoked PSCs from CA1 pyramidal cells. The absolute value on peak amplitude of NMDA-evoked PSCs during the last 10 minutes of the DL-APV (50μM) application significantly decreased when compared to both baseline period and CNQX administration: 23.9±4.9 pA, n=3, p<0.005, paired Student's t test.

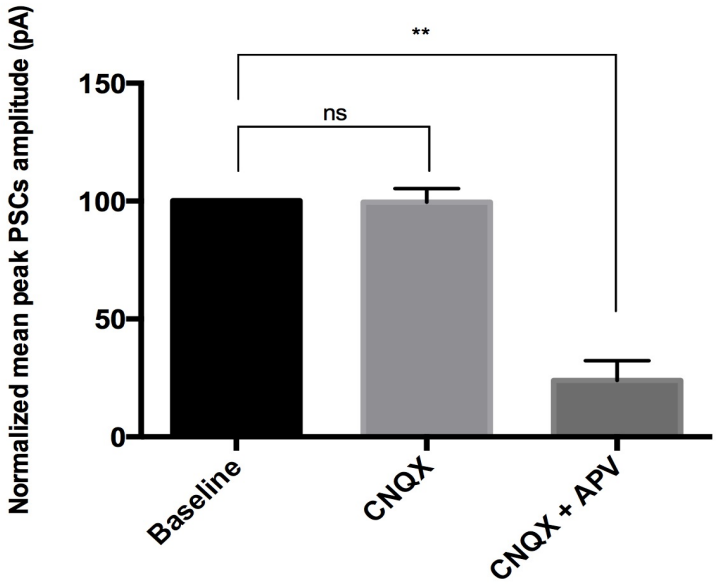

Figure 5.5.2 – NMDA receptor selective antagonist significantly reduces mean peak amplitudes of NMDA evoked-PSCs. Effect of NMDA receptor antagonist DL-APV (50μM) on mean peak amplitude of NMDA-evoked PSCs from CA1 pyramidal cells. Mean peak amplitude of NMDA-evoked PSCs during the last 10 minutes of the DL-APV (50μM) application significantly decreased when compared to the baseline period: 23.9±4.9 of baseline, n=3, p<0.005, under Student's t test (100% = 132,7±11,6 pA).

The graph displayed in figure 5.5.1 shows that the administration of DL-APV (50μM) caused a significant decrease in NMDA-evoked PSCs, when compared with a baseline period in which there was no NMDA antagonist present. Although there was a marked decrease in the measured postsynaptic currents, it is curious that those currents were not completely abolished.

DISCUSSION
6

The main findings in the present work were that A_{2A} adenosine receptors facilitate NMDA receptor activity in CA1 hippocampal pyramidal neurons. The results obtained in this investigation clearly suggest that the administration of the selective adenosine A_{2A} receptor CGS 21680 facilitated the excitatory responses mediated by NMDA receptor activity. This is a not yet described modulatory effect of adenosine A_{2A} receptors upon NMDA receptor activity in CA1 neuronal population. Given the ambivalent roles played by NMDA receptors in neuronal cell death processes, synaptic plasticity phenomena and correct development of the synapses, a full understanding of NMDA receptor activity regulation is extremely important.

6.1 - Modulatory effect of adenosine A_{2A} receptor upon NMDA-evoked postsynaptic currents

Adenosine is a neuromodulator known to influence the action of ionotropic glutamate receptors such as NMDA receptors (Sebastião & Ribeiro, 2000). Data obtained in recent investigations about the modulatory actions of A_{2A} receptors upon NMDA receptor activity it is still very scarce, even contradictory. In contrast to the well-known inhibitory ability of A_1 receptors to control excitatory synaptic transmission, adenosine A_{2A} receptors facilitate synaptic transmission (Cunha *et al.* 2008).

When compared with a baseline period free of agonist administration, the peak amplitude of NMDA-evoked PSCs markedly increased when A_{2A} receptors were activated with CGS 21680. The fact that upon washout of the agonist the peak amplitude of the currents significantly decreased, supports the conclusion that this

effect was indeed caused by the action of the selective A_{2A} receptors agonist CGS 21680. Furthermore, when the selective A_{2A} receptor antagonist SCH 58261 was administrated previously to CGS 21680, the facilitatory effect mediated by the agonist upon the registered NMDA-evoked currents was abolished. This indicates that the previous administration of a competitive antagonist resulted in the binding of the antagonist to the receptor allosteric sites of the A_{2A} receptors occupying the available binding sites. Ultimately this resulted in the dampening of the agonist-mediated response that was previously induced by CGS 21680 administration.

6.2 – A presynaptic facilitation in the release of glutamate or a postsynaptic modulatory interaction between receptors?

Given that the actions of CGS 21680 in pyramidal cells are linked with excitatory effects mediated by A_{2A} receptors located both at presynaptic and postsynaptic locations (Li & Henry, 1998), it seems needed to debate if this is a reflex of a presynaptic increase in neurotransmitter release, induced by the A_{2A} agonist CGS 21680 presynaptic facilitatory action upon glutamate release (Lopes *et al*. 1999) or, on the other hand, reflects a postsynaptic interaction between NMDA receptors and A_{2A} receptors located at the postsynaptic cell.

It is rather unlikely that the results reported in this work occur due to a presynaptic facilitation in glutamate release. First of all, the use of Tetrodotoxin, commonly known as TTX, allowed the blockade of action potential generation and all the spontaneous neuronal activity. By binding to the fast sodium voltage-gated channels located in the membrane of the nerve cells, TTX essentially prevents the firing of any affected nerve cell by blocking the channels used in the process. By using TTX in these experiments it was guaranteed that the presynaptic release of neurotransmitter was hindered, since neurotransmitter release is triggered by the arrival of an action potential. TTX blocks the possibility of having action potentials and therefore it is impossible for the presynaptic cell to produce the necessary calcium influx of calcium ions through voltage-dependent calcium selective ion channels. It would be these calcium ions that would bind to the proteins of the synaptic vesicle, allowing the vesicles to fuse with the presynaptic cell membrane and to release the neurotransmitter. Even with the presence of CGS 21680 in all the surrounding environment of the pyramidal cell under study by its superfusion in the bath solution,

it would impossible for the agonist to augment the release of neurotransmitter and hence increase NMDA receptor activity exclusively through presynaptic glutamate release facilitation.

The experimental protocol was designed to minimize the possibilities of presynaptic influences. Indeed, currents were being evoked through pressure application of NMDA, therefore bypassing activity dependent neurotransmitter release. Together with the use of TTX it becomes highly unlikely that NMDA receptor increased their activity due to an exacerbation in presynaptic glutamate release.

It seems likely that the facilitatory effect induced by the selective adenosine A_{2A} agonist upon NMDA-evoked PSCs in CA1 pyramidal neurons occurred due to a postsynaptic interaction between NMDA and A_{2A} receptors. Being so, these results must be interpreted together with other postsynaptic cross talks between modulatory adenosine receptors and NMDA glutamate receptors.

6.3 – Postsynaptic interaction between adenosine A_{2A} receptors and NMDA receptors

The postsynaptic interactions between adenosine A_{2A} receptors and NMDA receptors discovered until now differ accordingly to the hippocampal area in which they are described. Besides that, these interactions are not always straightforward, in the sense that they can be contradictory to what is expected. Clearly this subject needs more research to fully comprehend the mechanism that underlie the modulatory actions of A_{2A} receptors upon NMDA receptor mediated currents and the consequences of this particular neuromodulatory interaction.

In the striatum, it is known that the activation of A_{2A} receptors through agonist administration leads to an inhibition in the conductance of NMDA receptor channels. However, this only appears to happen in Mg^{2+} free environments, meaning in situations where NMDA receptors are not blocked by the presence of extracellular Mg^{2+}. In fact, when NMDA receptors are blocked, the predominant modulatory action of adenosine A_{2A} receptors is to presynaptically inhibit the release of GABA (Wirkner *et al*. 2004). It is important to refer that in the set of experiments now reported, Mg^{2+} was also withdrawn from the bath solution in all the experiments, since extracellular Mg^{2+} is known to inhibit NMDA receptor at resting membrane potentials. However,

in contrast of what occurs in the striatum, the A_{2A} receptor agonist significantly augmented the currents mediated by NMDA receptors in CA1 pyramidal neurons. If the facilitation mediated by A_{2A} receptors upon NMDA-evoked postsynaptic currents when there is no Mg^{2+} present in the cellular environment constitutes a mechanism exacerbating the possible deleterious actions of NMDA receptor activity concerning excitotoxic insults at the hippocampus is still elusive. Alternatively, facilitation of NMDA receptor activation may facilitate synaptic plasticity processes at the CA1 hippocampal area. Regardless, it is really intriguing that in situations favourable to NMDA receptor activity (no Mg^{2+} present to block their activity) A_{2A} receptors seem to display a further facilitatory effect over activation of NMDA receptors.

As I show here, A_{2A} receptor selective agonist administration resulted in the increase of NMDA-evoked PSCs. Adjunctively with the circumstance that there was no Mg^{2+} in the cellular environment and that the co-agonist was also present, ultimately this could mean that the modulatory facilitation effect of A_{2A} upon NMDA receptor activity would result in deleterious consequences for the cell, due to NMDA receptor over activation. However, the localization of the NMDA receptors being stimulated is crucially important to determine if NMDA receptor activation results in excitotoxic insults or synaptic plasticity. As referred in the introduction, more than the amount of calcium ions *per se* that flows through NMDA receptors, the influx of calcium ions into the postsynaptic cell through NMDA receptor located extrasynaptically is the most important factor determining the consequences of this increase in NMDA receptor activity (Hardingham *et al.* 2002). The influx of Ca^{2+} through synaptic NMDA receptors is well tolerated by the neuronal hippocampal cells. Additionally, it is associated with mechanisms that activate genomic processes that can make neuronal cells more resistant to apoptosis and oxidative processes (Hardingham *et al.* 2002).

There is also the question if this increase in NMDA receptor activity induced by A_{2A} receptor agonist administration could contribute to forms of LTP dependent on NMDA receptor activity in CA1 pyramidal neurons. In fact, CA1/CA3 LTP forms are mainly dependent on NMDA receptor activity (Sebastião & Ribeiro, 2009). However, some investigations have found that at CA1 hippocampal region, adenosine A_{2A} receptors activation is responsible for the induction of a LTP form that is independent of NMDA receptors (Kessey & Mogul, 1997). Curiously, knowing that at the mossy fibers/CA3 LTP forms are mainly independent on NMDA receptor

functioning (Sebastião & Ribeiro, 2009), some results show that, in the synapses localized between CA3 regions and mossy fibers synapses, the activation of A_{2A} receptors through agonist administration is related with the induction of a form of LTP mediated by postsynaptic NMDA receptors that spares AMPA currents. This form of LTP requires a simultaneous activation of NMDA receptors, mGluR5 receptors as well as A_{2A} receptors that ultimately lead to intracellular elevations in Ca^{2+} concentrations inside the postsynaptic neurons, triggering the LTP (Rebola *et al.* 2008). These two forms of LTP later described are somehow contradictory to what is characteristic in those hippocampal areas. It therefore appears that A_{2A} receptors are linked with secondary forms of plasticity in the hippocampus (Sebastião & Ribeiro, 2009). To understand if the increase in NMDA receptor activity mediated by adenosine A_{2A} receptors activation could lead to NMDA-dependent forms of LTP in CA1 pyramidal areas would constitute an important step to the work now reported.

6.4 – Contribution of extrasynaptic vs. synaptic NMDA receptor population for the observed effect

Glutamate excitotoxicity is identified as a key component in many neurodegenerative and neurologic diseases (Xu *et al.* 2009). NMDA receptors are the main responsible agents for the excessive and neurotoxic increase of Ca^{2+} inside the neuronal cell. In situations in which the postsynaptic cell is exposed to high levels of glutamate, it can lead to an over activation of NMDA receptors. Ultimately, It would lead to a significant increase in Ca^{2+} concentration inside the postsynaptic neuron (Xu *et al.* 2009). Ion calcium entrance through NMDA receptors is especially efficient in causing neuronal cell death, when compared to its entrance through other ionic channels (Hardingham & Bading, 2010). On the other hand, NMDA receptor activity can also be linked to molecular pathways favouring neuronal survival and synaptic plasticity processes (Xu *et al.* 2009).

Given the characteristics of the procedure designed for this investigation, it seems more likely that the NMDA-evoked PSCs were being mediated mainly by extrasynaptic NMDA receptors. First of all, it is important to remind that, in the young-adult brain, three-quarters of the NMDA receptors are extrasynaptically located in immature hippocampal neurons (Hardingham & Bading, 2010). Although there is an increase in synaptic located NMDA receptors throughout brain

development, in the adult brain the major part of these receptors are still extrasynaptically located (Hardingham & Bading, 2010). The pressure stimulation procedure used to directly stimulate the activity of NMDA receptors (*puff* stimulation with NMDA) creates a condition in which the agonist is applied to the entire surrounding cellular environment and not to specific regions or populations (in fact, the stimulation electrode is often placed above the registered neuron in order to assure that the *puff* affects all the cell). This way, and knowing that in the adult rat brain there higher amount of extrasynaptic NMDA receptors compared to synaptic ones, it seems highly probable that the NMDA-evoked PSCs were mainly mediated by extrasynaptic NMDA receptors. However, it would be necessary to create another experimental condition to fully understand this point.

There are two main ways of achieving this goal in electrophysiological procedures. Through afferent stimulation it could be possible to stimulate only the synaptically located NMDA receptors, therefore studying their contribution to the modulatory effect caused by A_{2A} agonist application. By changing the protocol used in this investigation, it could be possible to electrically stimulate the afferent nerves of the selected pyramidal neuron. The electrical stimulation of the afferent nerves of the presynaptic neuron would result in an increase in transmitter release. Glutamate is the main excitatory neurotransmitter and increases in glutamate release due to the electrical stimulation of the presynaptic neuron could activate the NMDA receptors located only in the postsynaptic cell and not the extrasynaptic NMDA receptor population. Measuring the NMDA-evoked PSCs in synaptic NMDA receptors population (under AMPA/kainate receptor blockade with a selective antagonist) and subsequently adding adenosine A_{2A} receptor agonist CGS 21680, would allow understanding if the modulatory effect still occurs when only synaptic NMDA population is being activated and if the effect is smaller than the one being registered. Although this experimental design could exclude some of the difficulties created by pressure stimulation procedures, it has serious limitations that are difficult to control. For instance, if the electrical stimulation of the presynaptic neuron was not perfectly adjusted, it could result in a spill over of glutamate. The excessive concentration of glutamate could migrate to the extrasynaptic areas of the postsynaptic neuron and therefore activate extrasynaptically located NMDA receptors.

Another way to selectively control the activity of a specific NMDA receptor population is to use memantine, a drug that preferentially blocks extrasynaptic over

synaptic NMDA receptor currents in the hippocampus (Xia *et al*. 2010). At micromolar concentrations (1-10 μM), memantine has the particularity of preferentially block extrasynaptic NMDA receptor activity rather than synaptic NMDA receptor currents in the same neuron (Xia *et al*. 2010). This tool is of particular interest to achieve the subsequent goals in this investigation. Memantine superfusion in the bath solution would allow the maintenance of all the procedure apparatus. NMDA receptor activity could continue to be evoked through pressure stimulation with direct agonist NMDA. This way, the modulatory effects of A_{2A} receptor agonist CGS 21680 upon NMDA-evoked PSCs could be attained in a condition were memantine was superfused in the bath solution and in a memantine-free condition, which would be used as a control condition. This would allow understanding the relative contribution of extrasynaptic NMDA receptor population to the modulatory effect caused by adenosine A_{2A} receptor agonist. In the eventuality that memantine causes a significant decrease in NMDA-evoked PSCs, one could argue that the effect occurred due to an interaction between A_{2A} receptors and extrasynaptic NMDA receptors (as predicted in this investigation). This could mean that the modulatory A_{2A} receptor effect upon NMDA receptor activity uncovered in this work could ultimately result in deleterious consequences for the neurons.

6.5 – The role of adenosine A_{2A} receptors and metabotropic glutamate receptors 5 (mGluR5) in NMDA-dependent forms of LTP in CA1 hippocampal areas

It is already describe that adenosine A_{2A} receptors are essential for a form of LTP that is dependent of NMDA-evoked excitatory postsynaptic currents at synapses located between the mossy fibers and CA3 pyramidal cells. This form of LTP does not demand the contribution of AMPA generated postsynaptic currents and it is likely induced and expressed at a postsynaptic level. It requires an elevation in Ca^{2+} concentration inside the postsynaptic cell and, for that, it is essential a co-activation of NMDA receptors and mGluR5 receptors. In addition, it is also necessary to activate adenosine A_{2A} receptors, which means that this is not a tonic effect, requiring synaptic stimulation (Rebola *et al*. 2008). The co-activation of NMDA receptors and mGluR5 receptors is a particular efficient way of increasing the concentration of Ca^{2+} inside the postsynaptic cell, having into account that the increase in Ca^{2+} concentration is the triggering element for LTP of NMDA-EPSCs, it seems that A_{2A} receptors, NMDA

receptors and mGluR5 receptors act synergistically to promote the durable potentiation of NMDA-EPSCs (Rebola *et al.* 2008). Accordingly, it is know that the co-activation of A_{2A} and mGluR5 receptors positively modulates NMDA receptor function (Tebano *et al.* 2005). In fact, the pharmacological activation of both NMDA and mGluR5 receptors potentiates NMDA-EPSCs inducing a long-lasting enhancing of excitatory synaptic transmission (Kotecha *et al.* 2003). This way, it looks like that these three types of receptor are necessary for the induction of NMDA-ESPCs LTP.

Interestingly, it is known that the activation of metabotropic receptors with selective agonists potentiates cAMP responses mediated by receptors that are positively coupled to adenylyl cyclase, such as A_{2A} receptors (Alexander *et al.* 1992). It seems that adenosine A_{2A} receptors and mGluR5 receptors are intimately connected and that their synergetic action modulates several processes. It would be interesting to investigate if the mGluR5 play some kind of role in experimental conditions in which adenosine A_{2A} receptors are modulating the activity of NMDA-evoked PSCs. As it was mentioned before, understanding if a simultaneous activation of A_{2A}, mGluR5 and NMDA receptors could be responsible for a form of LTP dependent on NMDA-EPSCs in CA1 pyramidal cells would be extremely interesting and could expose an effect not yet described.

6.6 – DL-APV did not cancel NMDA-evoked PSCs completely

Somehow unexpected was the lack of ability of DL-APV to completely cancel NMDA-evoked postsynaptic currents. DL-APV is a selective NMDA antagonist that competitively inhibits glutamate binding to the allosteric site of NMDA receptors. The utilization of this drug functioned as an important control to assure that the measure postsynaptic currents were exclusively mediated by NMDA receptors. Being a competitive, selective and efficient NMDA receptor antagonist, it was expected that the amplitudes of PSCs would be fully blocked by APV. Furthermore, taking into account that DL-APV was superfused in the presence of competitive AMPA/kainate receptors antagonist CNQX, by adding DL-APV every source capable of emitting PSCs in response to a pressure stimulation of NMDA or the presence of glutamate was blocked with an antagonist. Therefore, the expected result was to see the PSCs decrease to levels really close to zero or even to be completely cancelled.

There were some time limitations in this project that did not allow the increase in number of experiments using this APV. It would be relevant to increase the number of experiments, replicating all the procedure and ascertain if the NMDA-evoked PSCs are completely cancelled in the new experiment. Indeed, in in two of the three experiments considered, NMDA receptor-evoked currents were almost completely blocked and in the other one these currents reduced significantly. Being so, there are reasons to believe that this result could be replicated in further experiments. The concentration used in DL-APV should be increased. There are tow isoforms of the antagonist APV, the DL-APV (the one used in this investigation) and D-APV. The active isomer of APV is considered to be the D configuration but racemic chemical mixtures have created the mixture of D- and L- isomers, such as DL-APV, a racemic mixture between equal amounts of enantiomers of the chiral molecule. The chiral form DL-APV is relatively less active and specific than D-APV, and normally is used in smaller concentrations to fully block NMDA receptor activity. It may have happen that the concentration used in the preparation of DL-APV solution was not enough (50μM) when considering the experiment conditions. It is important to remind that the activity of NMDA receptors was stimulated through direct application of NMDA in a significant concentration (150μM). Also, the presence of glycine in both bath solution and rest solution, adjunctively with the extracellular Mg^{2+} withdrawn from the bath solution, were used to increase the probability of NMDA receptor activity making it more difficult for DL-APV to block NMDA receptor activity. Given that the pressure system is sending NMDA directly to the cell soma, this direct agonist will actively compete with the antagonist for the allosteric sites in the NMDA receptor. With the procedure configuration, probably it would have been more adequate to double the DL-APV concentration to 100 μM.

Nevertheless, in two of the three experiments considered, the reduction in NMDA-evoked PSCs was notorious, displaying current values close to zero. CNQX and TTX application in these experiments is really important because it allowed to discard the contribution of other glutamate receptors for the measured currents as well as a possible presynaptic facilitation in glutamate release that could augment the activity of these receptors. Moreover, previous experiments conducted at the host lab discard the possibility of the setup having some interference in the evoked currents. The *puff* stimulation electrode could in fact create some friction in the recording cell and therefore be responsible for the evoking of any current. However, this was

previously controlled did not occur. This way, it can be discarded a possible physical damaged caused by the electrode upon the recording neuron.

Summarizing, in future experiments it is necessary to replicate the experiments using a higher concentration of DL-APV to observe if with the new concentration the currents mediated by NMDA receptor activity are completely cancelled.

GENERAL **C**ONCLUSIONS

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The present results provide in first hand direct evidence that in CA1 pyramidal neurons the selective adenosine A_{2A} receptor agonist CGS 21680 is responsible for a modulatory effect that potentiates NMDA receptor activity, increasing the postsynaptic currents mediated by these receptors. This effect seems to be exclusively mediated by the action of A_{2A} receptors upon NMDA receptors.

Further studies are necessary to discriminate between the relative sensibility of synaptic and extrasynaptic NMDA receptor population to the effect of A_{2A} receptor agonist. This could allow foreseeing the functional consequences of the A_{2A} receptor mediated modulation, given the different role of synaptic and extrasynaptic NMDA receptors for neuronal functions.

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8

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